"Why do individuals respond differently to infection with the same pathogen?" "Why is the response to vaccination with the same vaccine so different per person?" "Why do patients react differently to the same treatment?" Possible explanations for these differences are variable environmental factors, differences in immune status, or pathogen variation. Another likely answer to these questions is the topic of this thesis: "individuals are different" and in particular: "they carry different genetic factors". In this thesis we examine these genetic differences to learn more about pertussis.

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Genetics and genomics of B.pertussis Sander Banus 2008

Host genetics and genomics of Bordetella pertussis infection and vaccination

Sander Banus

Uitnodiging

Voor het bijwonen van de openbare verdediging van mijn proefschrift

Donderdag 5 Juni 2008
om 10.30 uur
in de aula van

Radboud Universiteit
Nijmegen
Comeniuslaan 2
6525 HP Nijmegen

Na afloop van de promotie bent u van harte welkom op de receptie

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Host genetics and genomics of *Bordetella pertussis*

infection and vaccination

Een wetenschappelijke proeve op het gebied van de

Medische Wetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Radboud Universiteit Nijmegen

op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,

volgens besluit van het College van Decanen

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donderdag 5 juni 2008

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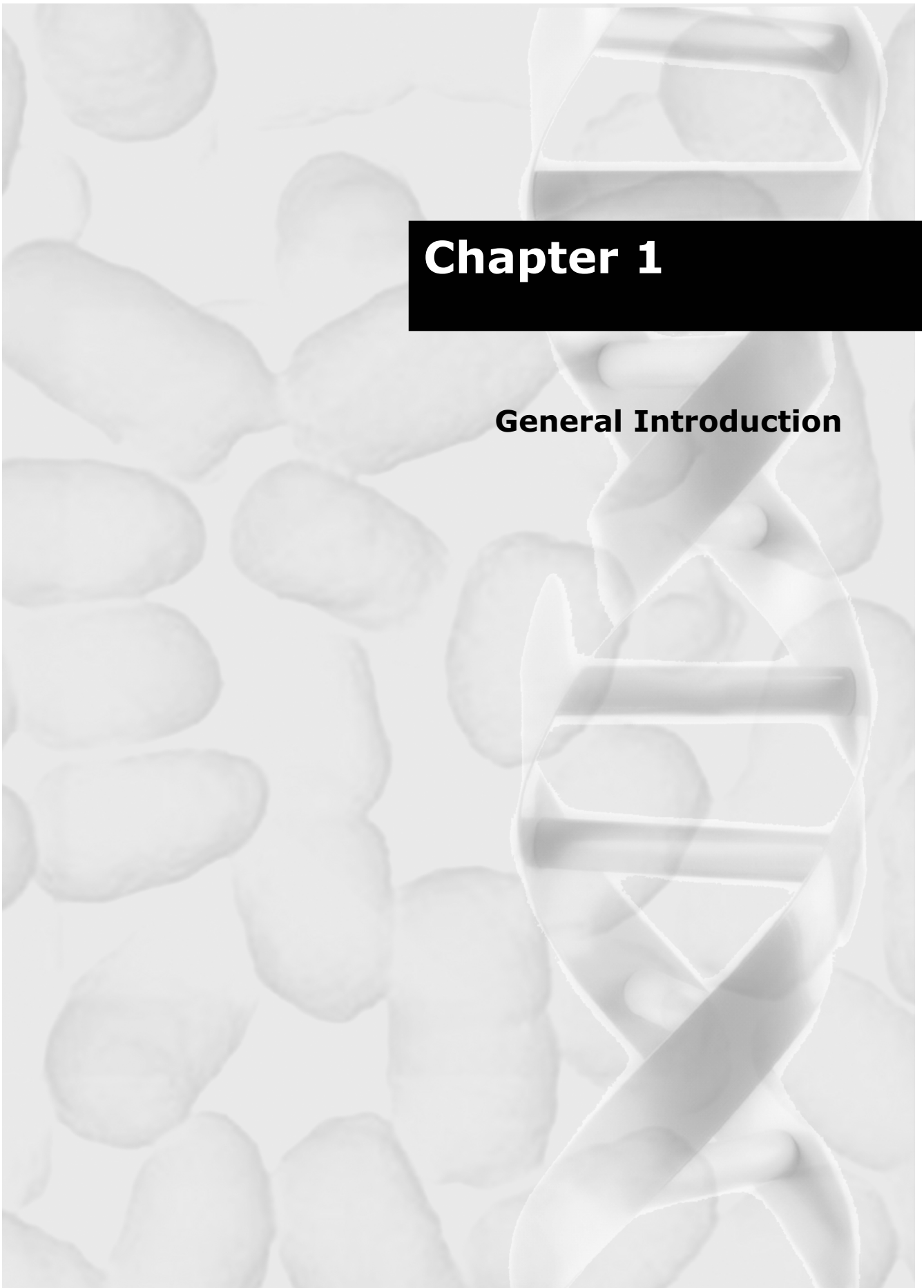

Chapter 1

General Introduction

1.1 Introduction

Whooping cough is a highly contagious infectious disease caused by the airway pathogen *Bordetella pertussis*. Since the introduction of the vaccine against pertussis in the 50's, morbidity and mortality have decreased drastically but the incidence has increased during the last decennia in most countries, including the Netherlands(20,21). In spite of world wide vaccination against pertussis, whooping cough is still endemic in most countries, causing 297,000 deaths annually(62). Especially young non-vaccinated children are at risk for major symptoms such cyanosis or even fatalities (62).

1.1.1 Symptoms

After a 7-10 days incubation period, the first characteristics of the disease manifest themselves by symptoms like a common cold, malaise, low grade fever and progressive cough. This first stage (catarrhal phase) lasts 1 to 2 weeks and is followed by the paroxysmal phase, characterized by the typical 'whooping' paroxysms often with cyanosis and vomiting. The paroxysmal phase begins gradually with prolonged and paroxysmal coughing that often ends in a characteristic inspiratory gasp (whoop). After 2-4 weeks the final convalescent period dawns, typified by diminished frequency and duration of the coughing periods (17,45,59).

1.1.2 Incidence

Despite high vaccination coverage pertussis is still endemic in The Netherlands with epidemic peaks every 2 to 3 years during the last decade. Since 1996 a large increase in the number of notifications, positive cultures, positive serologic diagnoses, and hospital admissions was observed (20,21). The incidence of pertussis rose suddenly from 1996 onwards to reach 58,3 reported cases per 100 000 population in 2004, compared with 2.3/100 000 on average from 1989 to 1995 (52,53). Also in several other European countries, as well as Canada, The United States and Australia, a re-emergence of pertussis has been observed as is illustrated in figure 1 (18,19).

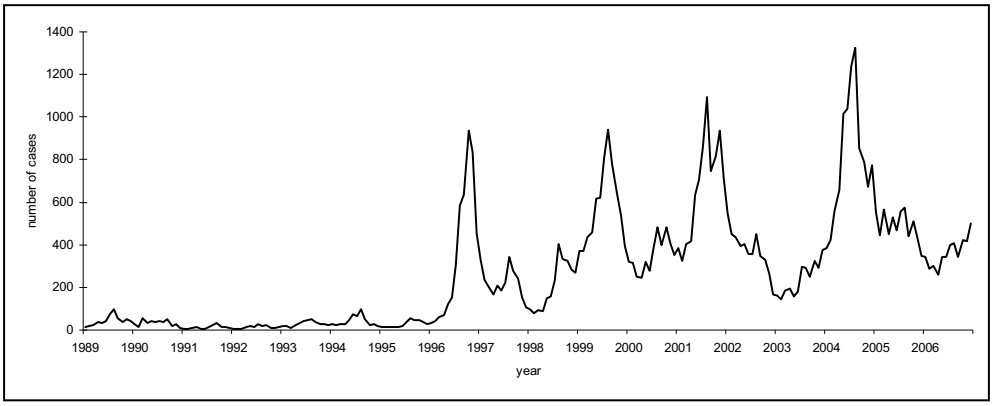

Figure 1: The number of reported cases (legal notifications) of Pertussis in the Netherlands (19).

Waning immunity, increased reporting, improved diagnosis, and adaptation of the bacterium have been proposed to explain this re-emergence. While pertussis is commonly known as a childhood disease, nowadays there has been an increase in the incidence of pertussis in adolescents and adults (3,59).

1.2 *Bordetella pertussis*

The genus *Bordetella* encompasses nine known species which vary in the ability of infecting different hosts, and by the expression of different virulence factors (23,24). Besides *B. pertussis* only *Bordetella parapertussis, Bordetella bronchiseptica* and *Bordetella holmesii* have been associated with respiratory infections in humans and other mammals (30). The primary focus of this thesis is *B. pertussis*, and other *Bordetellae* are therefore left aside.

1.2.1 The BvgAS system.

B. pertussis can express a wide variety of virulence factors, controlled by the Bordetella virulence genes (Bvg) control system. This system consists of two components, a DNA-binding response regulator (BvgA) and a transmembrane sensor kinase (BvgS). The BvgAS system responds to environmental stimuli by controlling the expression of the virulence factors. *B. pertussis* can exist in three different phenotypes, Bvg^+, Bvg^i and Bvg^- respectively. Bvg^+ is the virulent stage of *B. pertussis*, in which several virulence factors are expressed by which the bacteria can colonize the respiratory tract of the host. The Bvg^- is the a-virulent phase, known as the starvation mode. In this stage the majority of the virulence factors are down-regulated, whereas other genes (e.g. motility genes) are up-regulated.

The Bvg intermediate phase (Bvg^i) is hypothesized to be important for respiratory transmission and is characterized by the expression of a subset of Bvg^+ phase-specific factors (30,45,46). The different virulence factors will be addressed in the next paragraph.

1.2.2 Virulence factors

B. pertussis expresses various virulence factors (*Figure 2)* with different functions to facilitate infection and to induce pathology of the host. The most important virulence factors are summarized below.

The exotoxin Pertussis toxin (PT) is the primary component of acellular pertussis vaccines (ACV). PT is a member of the A-B (A, active domain; B, binding) bacterial toxin superfamily and consists of five different subunits (S1 to S5) with different functions. The S1 protein is the A subunit which has ADP ribosyltransferase activity. Together, the subunits S2-S5 constitute the B subunit which facilitates the attachment and entry of pertussis toxin into host cells (30,61).

Filamentous hemagglutinin (FHA) is a surface-associated and secreted protein required for tracheal colonization. FHA binds to galactose residues on a sulfated glycolipid called sulfatide which is very common on the surface of ciliated cells. FHA is highly immunogenic and one of the main components of acellular pertussis vaccines (ACV) (45,61).

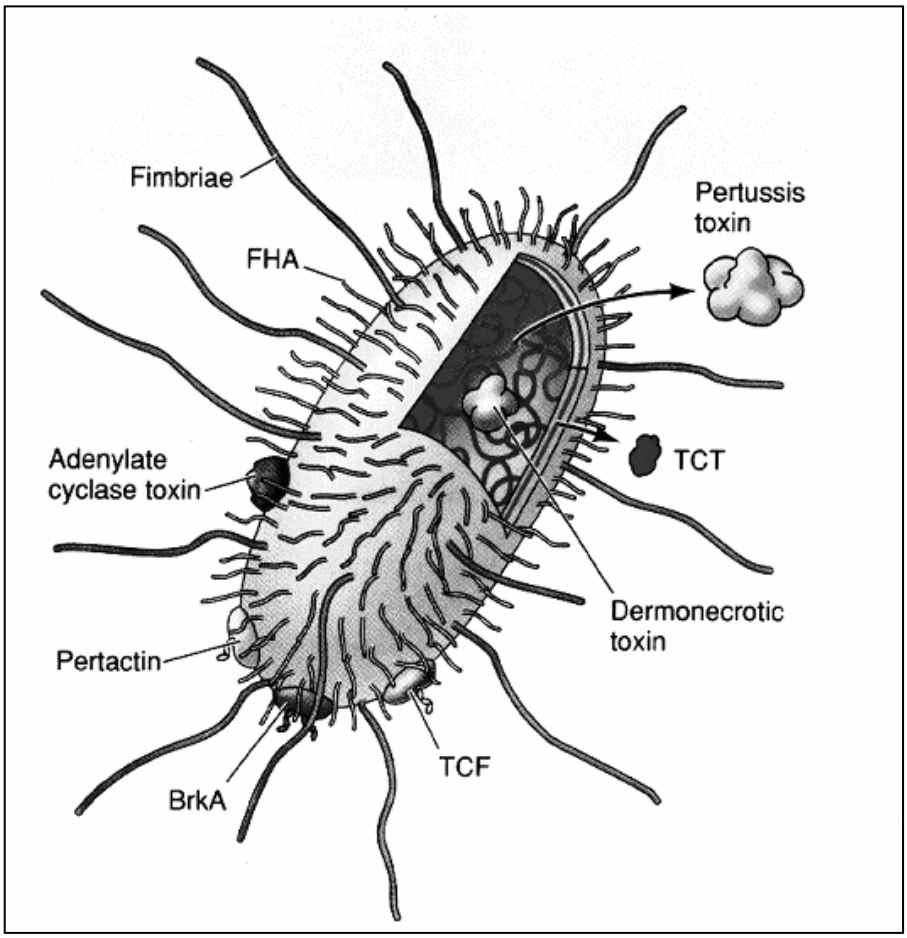

Figure 2: Virulence factors of Bordetella pertussis *(Adapted from A.Weiss)*

Fimbriae (FIM) are long filamentous polymeric proteins on the surface of the bacterium which have been proposed to mediate attachment of *Bordetella* to respiratory epithelium required for persistent tracheal colonization. FIM is one of the components of some ACV's for its ability to induce protective immunity to infection (45,61). The surface protein Pertactin (PRN) belongs to a class of auto transporter proteins that undergo autoproteolytic processing. PRN has been shown to play a role in adhesion of the bacterium to monocytes. Antibodies against PRN have been shown to correlate with clinical protection against pertussis. PRN is one of the components of some ACVs (35).
The endotoxin Lipopolysaccharide (LPS) is the main component of the outer leaflet of the outer membrane of *B.* pertussis. By activating Toll-like receptor 4 (Tlr4) signaling pathway, LPS promotes the secretion of pro-inflammatory cytokines, such as tumor necrosis factor in many cell types, but especially in macrophages. LPS is very reactogenic and pyrogenic, and is therefore unwanted in vaccines (30).

1.2.3 Pathogenesis

Pathogenesis of *B. pertussis* infection is characterized by colonization and proliferation of bacteria on the ciliated mucosal cells, resulting in damage of the respiratory epithelium, and an acute increase in the levels of inflammatory cytokines resulting in cellular infiltrate in the alveolar spaces (45,49,51).
Circulating polymorphonuclear leukocytes (PMN's) are rapidly recruited to the lungs to bind and ingest *B. pertussis*, subsequently killing the bacteria by a combination of reactive oxygen and granule components. Finally, the PMN's undergo apoptosis (44). Toll-like receptor (TLR, §1.3.2) ligands such as bacterial lipopolysaccharide (LPS) are critical components for the recruitment and activation of PMN's (43). Serum antibody–mediated clearance of *B. pertussis* also requires a TLR-induced early recruitment of PMN's. However, in mice it was shown that pertussis toxin limits this rapid serum antibody–mediated clearance by inhibiting PMN recruitment (43)

1.3 Immunity

1.3.1 Innate and adaptive immunity

The immune system can roughly be divided in two types, the innate and the adaptive immune system. The adaptive or acquired immune system evolves during the multiple infections in life, and develops epitope-specific antibodies and the B- and T-cell memory systems. The innate immune system (or first-line defense) responds more quickly compared to the adaptive immune response, but does not develop a memory response as the adaptive response does. The innate immune system responds to pathogens in stead of specific epitopes. These pathogens are recognized by receptors with a broad specificity that can recognize pathogen-associated molecular patterns (PAMS). The major class of receptors that recognizes these PAMPS are Toll-like receptors.

1.3.2 Toll-like receptors

There are different Toll-like receptors (TLR), with an identical intracellular domain known as Toll/IL-1R (TIR) domain (47,48). The TLRs differ especially in their ligand specificity: Lipoprotein is recognized by TLR1 or TLR6, Peptidoglycan is the agonist for TLR2, Flagellin are recognized by TLR5, Imidazoquinolines (anti-viral compounds) can activate TLR7, TLR9 recognizes CpG DNA, dsRNA is the ligand for TLR3 and Lipopolysaccharide (LPS) is recognized by TLR4 (1).

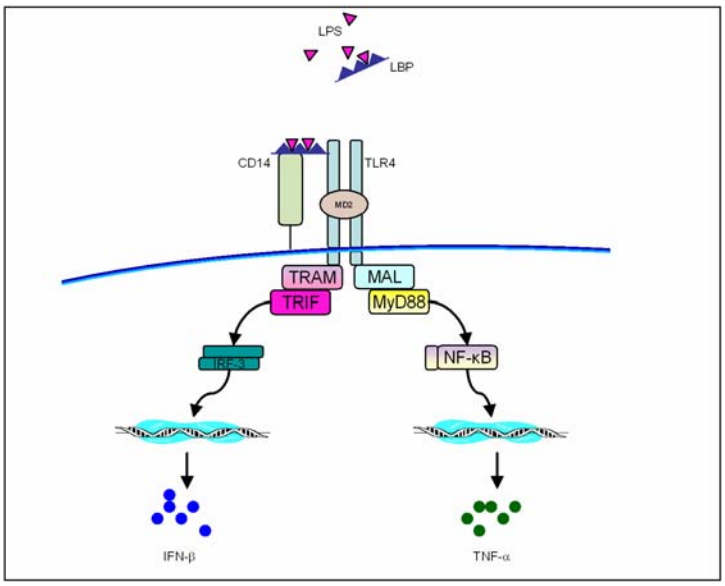

Figure 3: Toll-like receptor 4 signalling.

1.3.3 Toll-like receptor 4

Toll-like receptor 4 (TLR4) was the first TLR to be described and was originally designated human Toll (47,48). It was shown that TLR4 directly influences innate immunity by studies in Tlr4 defective mice (12,50).

The innate receptor for LPS is situated on the outer membrane of antigen presenting cells and B-cells (*Figure 3,* macrophages, dendritic cells and natural killer cells). LPS binds to the LPS-binding protein (LPB) that circulates in blood. This complex binds to the co receptor of Tlr4, cluster of differentiation 14 (CD14). When this complex binds to the MD2-Tlr4 complex, Tlr4 is activated, triggering a down-stream signaling (29,32,39).

1.4 Vaccination

The reasons to vaccinate against pertussis are prevention of the disease (especially children younger than 1 year of age are at risk for developing major symptoms such cyanosis or even fatalities), prevention of infection of idividuals and decreasing the number of carriers to prevent the spreading of bacteria.

Since the introduction of the whole cell pertussis vaccination in the Netherlands, mortality reduced drastically. Since January 1999 the primary vaccination for pertussis has been advanced and children are vaccinated at the age of 2, 3, 4 and 11 months, instead of 3, 4, 5 and 11 months from that time. Since the whole cell pertussis vaccines contain strong immunogens like Ptx and LPS, they are rather reactogenic, and several side-effects were reported. During the last decades, the incidence of pertussis increased, the public opinion changed, and the Dutch government decided to switch from whole-cell pertussis vaccine (WCV) to acellular pertussis vaccine in 2005 (ACV) (34,62).

1.4.1 WCV

In 1952, whole cell pertussis vaccination was included in the Dutch vaccination program. Whole cell pertussis vaccine consists of heat-killed *B. pertussis*, and thus contains all epitopes of the bacterium, thereby inducing a broad immune response (34). Antigens are especially directed at the surface antigens PT, PRN, FIM. The serum titers of PT-IgG have been shown to correlate with protection against pertussis. Although LPS or endotoxin has adjuvant effects, also on other simultaneously administered vaccines (such as diphtheria), it can also induce strong side-effects (30).

1.4.2 ACV

As a result of these side-effects, children in the Netherlands are vaccinated with accellar vaccines since 2005 (21). ACV contains purified proteins of *B. pertussis*, by which a stronger, but narrower immune response is induced (15,16). There are several ACV's registered which differ in their composition of virulence factors, PT, FHA, PRN and FIM. The Dutch ACV comprises 3 components, PT, FHA and PRN. ACV induces less side-effects (less reactogenic) compare to WCV while the vaccine efficacy (induced protective antibody-titers against the specific epitopes) is good. However, although the vaccination coverage in the Netherlands is very good (approximately 95%), pertussis is still endemic in the Netherlands. Several approaches to reduce disease incidence and severity have been suggested, one of them being the improvement of existing aP.

1.4.3 Adjuvant

To enhance the effectiveness of vaccination, the vaccines are complimented with immune-stimulating agents known as adjuvants. An adjuvant is an agent that stimulates the immune system without having any specific antigenic effects itself. Adjuvants are important for enhancing the primary response and for inducing long-term memory. The Dutch wP is adjuvated with aluminium phosphate, the aP is adjuvated with aluminium hydroxide. When the vaccine combined with the adjuvant is administered, one of the functions of the adjuvant is that a granuloma is formed (11,27), out of which the vaccine-antigens are slowly released. This results in a prolonged stimulus. The use of adjuvants also reduces the amount of purified antigen required for successful immunization, thus making vaccine production more economical and more feasible (26). The use of aluminum as adjuvant is associated with local reactions in young children (38).

1.5 Susceptibility to infectious diseases

As described in the previous paragraphs, *B. pertussis* can express different virulence factors that have functions in establishing infection and disease. The success of infection also depends on different environmental factors, such as population density, as well as host factors such as immune status and host genetics. The genetic variability of the host plays a role in processes such as adhesion, specific and non-specific immunity, antigen presentation, and inflammation. The susceptibility or resistance to infectious diseases can be exploited to gain knowledge of infectious diseases and improve their treatment and prevention (13,41,42).

Generally, there are two approaches for the identification of host genetic factors that influence the course of an infectious disease: with or without a priori assumptions. In humans the identification of host genetic factors that play a role in complex diseases is difficult because of the small individual effects and the genetic heterogeneity of the population, therefore an approach without a priori assumptions is very difficult. Biological processes of health and disease are nowadays often characterized by the involvement of biological pathways. A 'pathway' is defined as the mutual coherence of factors (such as proteins, DNA) and the sequence of communication between these factors. Most studies in humans focus on a specific pathway and study the association between single nucleotide polymorphisms (SNPs) of specific genes within such a pathway and a phenotypic parameter (42). In contrast, the mouse offers significant advantages as a model to study the effects of host genetics on infectious diseases without a priori assumptions, but the path from susceptibility locus to susceptibility genes requires intensive study and many animals (10,54). Further, established susceptibility genes in mice, should be extrapolated to the human homologues. Only recently new techniques have been developed by which host genetics can be studied in man by whole genome analysis using an array-based SNP assay with up to one million SNP's that are analyzed simultaneously. Excessive costs, multiple testing problems and the requirement of large cohorts are limiting factors at this moment, but these techniques will be more easily available in the near future (60).

1.5.1 Mouse models

To define the numerous susceptibility genes that have small but cumulative effects on a specific phenotype, an appropriate approach is to first identify them in a mouse model, and subsequently to study the role of their human homologues in humans (25,54). To map susceptibility genes in mice, several different strategies are available encompassing different mouse models as ´genetic tool´ to facilitate linkage studies (22). Different mouse models have a similar approach: by series of crossings between different parental strains of mice (the so-called background strain and the so-called donor strain), an offspring is generated containing specific chromosomal regions from the donor strain across the donor genome. The genome is genotyped with microsatelite markers or single nucleotide markers to locate the donor regions across the background genome. Examples of different mouse models are congenic inbred strains (CIS), chromosome-substitution strains (CSS), genome tagged mice (GTM) or recombinant congenic mice (RCS).

CIS are ´ordinary´ homozygous strains that are derived by series of crossings between two parental inbred strains (background strain and donor strain), with donor regions randomly spread across the background genome. CSS strains contain one chromosome from the donor strain, the remainder originates from the background strain (Figure 4a) (58). GTM can be considered as partial CSS. Each GTM line differs from the background strain only in a relatively short segment of one chromosome. Phenotypic differences between a GTM line and the background strain can be attributed to one or more genes on the differential segment of the chromosome (Figure 4b). RCS are homozygous strains that are derived from two parental inbred strains. Each RC strain (in a usual set of 20 or more) is homozygous, and contains a randomly spread set of ~12.5% genes from strain S and 87.5% genes from strain C (Figure 4c) (22,54). In this thesis CIS and RCS strategies were used for the identification of *B. pertussis* susceptibility loci in mice.

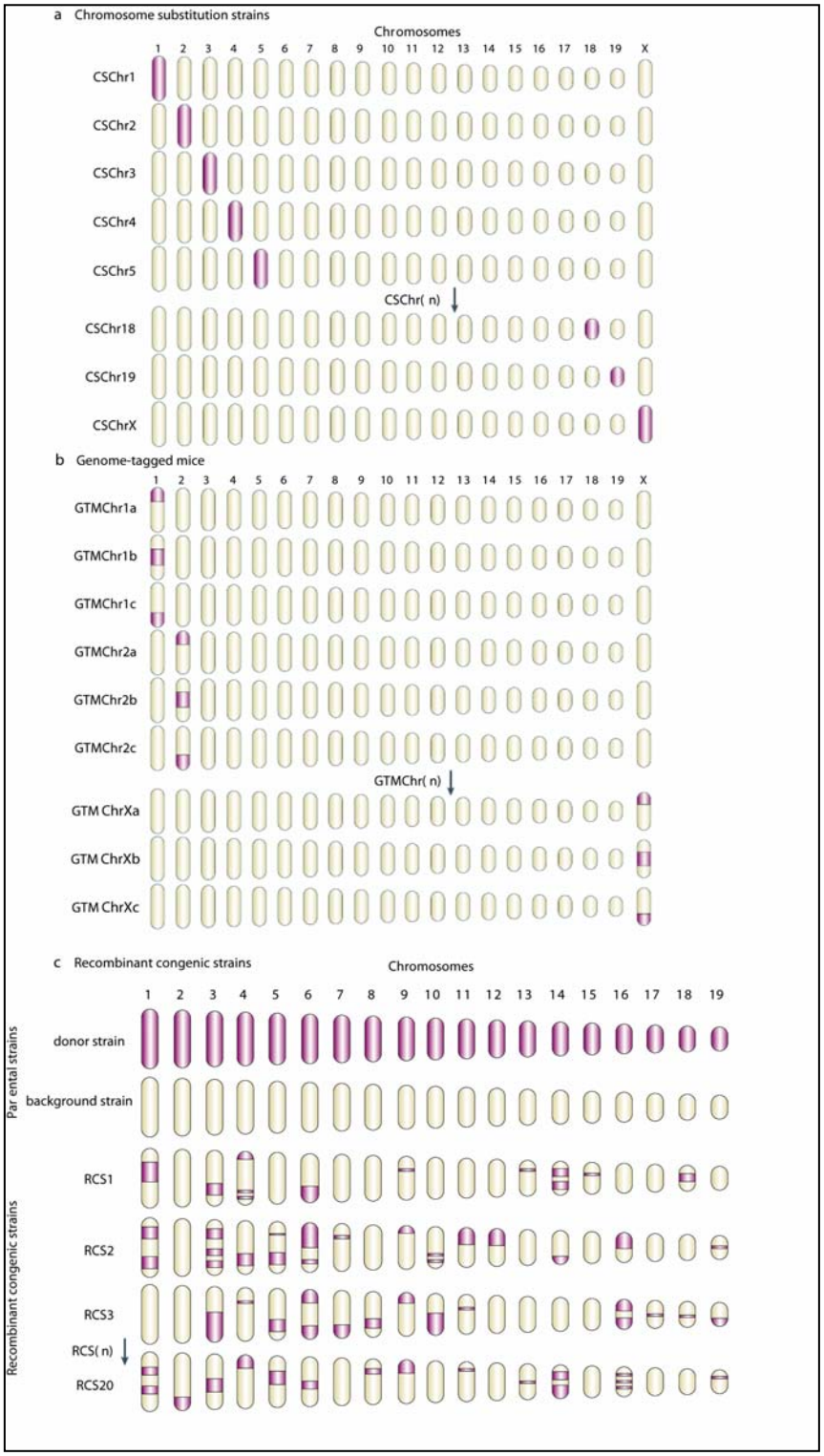

Figure 4: Tools to map Mouse susceptibility loci. ***a*** *| Chromosome substitution (consomic) strains. Each consomic strain contains one differential chromosome from the 'donor' strain; the remainder originate from the 'background' strain. Phenotypic differences between a consomic strain and the background strain can be attributed to one or more genes on the differential chromosome.* ***b*** *| Genome-tagged mice (GTM). Each GTM line differs from the background strain only in a relatively short segment of one chromosome. Phenotypic differences between a GTM line and the background strain can be attributed to one or more genes on the differential segment of the chromosome.* ***c*** *| Composition of recombinant congenic strains. Recombinant congenic (RC) strains are homozygous strains that are derived from two parental inbred strains. Each RC strain (in a usual set of 20 or more) is homozygous, and contains a random different set of ~12.5% genes from the donor strain and 87.5% genes from the background strain. Sex chromosomes are not shown in this example. Modified from Demant, 2003(22)*

To map individual susceptibility loci, mice are infected, and the relevant phenotypic parameter is determined (for instance colony forming units). Subsequently the mice are genotyped at marker loci (microsatellites or single nucleotide polymorphisms) that are polymorphic between the two parental strains. The correlation between the distribution of marker alleles and the susceptibility phenotype(s) allows susceptibility loci to be mapped to chromosomal locations (2,22,28,57).

1.5.2 Human studies

There is substantial evidence that host genetic factors play a major role in determining the outcome of infection with many pathogens (36). Genetic control of susceptibility to infectious diseases provides new tools for prevention and control of these diseases. Whole genome-based genetic approaches have the major advantage that no a priori assumptions about mechanisms of pathogenesis need to be made in these studies, and thus previously unrecognized pathways of disease susceptibility can be detected (55).

The classical approach for a human association study is the case-control study which is used to identify factors that may contribute to a medical condition by comparing the genotypes of a group of patients who have that condition with the genotypes of a group of control persons. For this approach, certain genetic variants of genes are selected a priori, usually from the disease pathway. This a priori selection is often called a hypothesis-based approach. The most frequently occurring form of genetic variation in the human genome is the single nucleotide polymorphism (SNP). SNPs are chromosomal positions at which more than one nucleotide can occur when genomes of different individuals are compared and that are present by definition in 1% or more in the population (note: when this chromosomal nucleotide variation is present in less than 1% of the population, it is called 'mutation' by definition). Most SNPs are located in non-coding regions (with little evolutionary pressure) such as intronic regions (between exons), in the non-coding regions between genes, or in 5' and 3' regulatory regions. SNPs in coding region (exons) can cause an amino acid substitution, or, despite the change in codon, encode the same amino acid, resulting in an unchanged protein. Although the functional relevance of non-coding SNPs is limited or unknown, it could still be very useful for the identification of susceptibility alleles in association studies, acting as a marker through linkage to another causal variant (haplotype analysis) (56) .

At this moment, array-based whole-genome genotyping assays are developed covering over one million SNPs to interrogate human genetic variation (33,37,40). Such assays make whole-genome association studies more feasible and have good prospects for dissecting the genetics of common diseases. They

currently face a number of challenges, including problems with multiple testing and study design, definition of intermediate phenotypes and interaction between polymorphisms (14).

1.6 Aims and outline of this thesis

The objective of this thesis is to acquire knowledge of genetic host factors that influence the pathogenesis of *B. pertussis* as well as host factors that influence the vaccination response against pertussis. By increasing these insights in host factors, new methods can be developed for treatment or prevention of whooping cough. Eventually, such insights may provide novel concepts in infectious disease control by extrapolation of the knowledge acquired for *B.* pertussis to other infectious diseases. This thesis can be divided in two parts, the first part describes the role of host genetic factors in the pathobiology of pertussis disease, and the second part focuses on the role of toll-like receptor 4 in the pathogenesis of, and the vaccination against pertussis.

In Chapter 2 we describe the identification of three novel susceptibility loci in mice. We designate these loci *Bordetella pertussis susceptibility loci-1, -2 and -3.* We identify these loci by using recombinant congenic strains of mice (7). In Chapter 3 we describe the expression profiles of pertussis infected mice. In this chapter we demonstrate that the expression of 1,841 genes is significantly changed upon *B. pertussis* infection. These genes are involved in various immune-related processes, such as the acute-phase response, antigen presentation, cytokine production, inflammation, and apoptosis (5). Chapter 4 describes differential gene expression between the C3H and HcB strains, the two strains of mice in which we identified the *Bps-1* locus. By this approach, we narrow down the number of susceptibility genes within this locus(8).

Toll-like receptor 4 is the receptor for lipopolysaccharide, one of the major components of the cell-membrane of *B. pertussis*. In Chapter 5 we demonstrate the significance of Tlr4 in genetic susceptibility and pathobiology of pertussis in mice. We show that functional Tlr4 is essential for an efficient (pro) inflammatory response; efficient clearance of bacteria from the lung; and reduced lung pathology. (9).

The role of Tlr4 in vaccination response to *Bordetella pertussis* in a murine model is demonstrated in chapter 6 (6) and in chapter 7 we show that TLR4 also plays an important role in the vaccine-induced immunity in children (4). We show that a Toll-like receptor 4 polymorphism is associated with the response to whole-cell pertussis vaccination in children (4). Chapter 8 gives an application of the acquired knowledge of the role of Tlr4 in pertussis vaccination. Here we describe that LPS analogs improve acellular pertussis vaccine (31).

Finally, in Chapter 9 the findings of the preceding chapters are summarized and discussed and future perspectives are presented.

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Chapter 2

Genetic control of *Bordetella pertussis* infection: Identification of susceptibility loci using recombinant congenic strains of mice.

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Abstract

Susceptibility to and severity of *Bordetella pertussis* infection in infants and children varies widely. The spectrum of clinical symptoms ranges from subclinical infection, mild disease, severe whooping cough, and death. The aims of this study were to examine genetic susceptibility in mice to *B.*pertussis, and to identify genetic loci in the mouse genome that are involved in susceptibility to *B.pertussis* infection. To this purpose we screened two sets of recombinant congenic strains (RCS) of mice, HcB and CcS, for differences in the numbers of bacteria in the lung seven days after inoculation. In both CcS and in HcB mice, a wide range in numbers of bacteria in the lung was found, suggesting that the course of infection is under multigenic control. From both RCS sets of mice, we selected one strain to identify possible susceptibility loci in F2 hybrid mice. The degree of lung colonization seven days post-inoculation in these F2 mice was compared with genetic markers by linkage analysis. We found three novel loci that are involved in the control of *B.pertussis* infection. One locus, designated *B.pertussis susceptibility locus 1* (*Bps-1*), was identified on chromosome 12. The presence of C57BL/10 genome on this locus in stead of C3H genome significantly decreased the number of *B.pertussis* bacteria in the lung. *Bps-1* has a dominant positive effect on the clearance of *B.pertussis* from the lung. The function of most genes in this region is unknown. Two other loci, *Bps-2* and *Bps-3* showed genetic interaction and are located on chromosome 5 and 11. We aim to identify the gene(s) in these regions which modify the susceptibility to *B.pertussis*.

Introduction

Bordetella pertussis is a gram-negative bacterium that causes the respiratory disease known as whooping cough or pertussis. Worldwide, this bacterial agent causes some 20–40 million cases of pertussis and an estimated 300.000 deaths each year (35).

The bacterium enters the airways via aerosol droplets and attaches to the epithelium of the upper respiratory tract. After a 7-10 days incubation period the first symptoms of the disease, which are similar to the common cold, may be observed. One to two weeks later more serious symptoms can occur, such as the typical 'whooping cough' (23). In spite of worldwide vaccination since the 50's, the incidence of pertussis is increasing again (4). Several causes have been suggested for the resurgence of pertussis, including improved diagnostics and surveillance, waning immunity, and the emergence of escape variants (27,28).

The clinical course of *B.pertussis* infection varies widely. Knowledge about host genetic and immunological factors that influence susceptibility and severity of the infection, may lead to the identification of new approaches for prevention or treatment of infectious diseases (14). Yet, knowledge about human genetic factors that influence *B.pertussis* infection is still very limited. A number of studies provides clues for the role of host genes in susceptibility to *B.pertussis* infection. The genetic make-up of mouse strains effected the immune response against *B.pertussis* (2,21,25). This was also confirmed in studies using the respiratory *B.pertussis* infection model in knock-out mice, where numerous genes, such as CR3,CD32,CD32,FcRγ, IFN-γ, IL-4 and Ig, have been knocked-out to establish their involvement in the pathogenesis of *B.pertussis* infection. (12,25). Recently a mutation in the Toll Like Receptor 4 (*Tlr-4*) was identified as a major factor that influences the course of *B.pertussis* infection in mice (13,22). In human cell lines, differences in expression between *B.pertussis*-treated and untreated cells have been found for a number of genes. Upregulated genes encoded cytokines, chemokines, anti-apoptotic factors, and Nuclear factor of κB (NF-κB), whereas downregulated genes encoded DNA-binding proteins and cellular adhesin molecules (3,30).

In general, to identify unknown genes involved in the course of complex diseases, quantitative trait loci (QTL) mapping studies in humans or animals have been used (7). A QTL is a polymorphic locus which contains alleles that differentially affect the expression of a continuously distributed phenotypic trait. QTL mapping is a phenotype-driven approach to identify genes affecting a phenotype. As such it permits the discovery of new genes and contrasts with gene-driven approaches, such as knock-out mice, which allow the study of genes with known function (5,9).

There are several ways to map a QTL. One approach, which reduces the genetic complexity of the mouse genome by ~90%, uses so called Recombinant

Congenic Strains (RCS) of mice (8). This approach also enables the identification of possible low-penetrance genes and their interactions (7,10,29). RCS are derived from two different inbred strains, the so called background and donor strain. After two backcrosses and intercrossing, a set of RCS is created, with each strain containing 12.5% of the donor genome differently distributed across the background genome (8). The approximate distribution of these chromosomal regions of the donor strain is called strain distribution pattern (SDP). A more detailed description, including an example of such an SDP was provided by P. Demant and coworkers(11,31).
The aims of this study were, first, to examine whether RCS of mice show genetic differences in susceptibility to *B.pertussis,* and, second, whether we could identify one or more genetic loci responsible for such differences. We used the number of bacteria in the lung one week after inoculation to define the phenotype, and microsatelite markers to define the genotype. Using this approach we identified a locus on chromosome 12, designated *B.pertussis susceptibility locus 1* (*Bps-1*), and 2 interacting loci on chromosome 5 and 11, designated *Bps-2* and *3,* which influence the number of bacteria in the lung one week after inoculation.

Materials and Methods

Experimental design

We examined the course of B.pertussis infection in twelve different CcS/Dem strains and twenty-one HcB/Dem strains. Approximately ten mice of each strain, i.e. 145 CcS and 170 HcB mice, were tested for the number of bacteria in the lung one week post-inoculation. Two F2 hybrid generations of mice generated from two different recombinant congenic strains, 211 (CcS4 x BALB/c) F2 mice and 230 (HcB28 x C3H) F2 mice, were subsequently phenotyped as described below.

Due to logistical limitations we inoculated maximally 100 mice per day and combined the results. To test the reproducibility of the infection model, on several days the experimental groups contained BALB/c control mice that were inoculated in the same way. The original RCS mice were examined in four experiments, the F2 hybrid mice were examined in eight experiments.

Animals.

Only female mice were used for the infection experiments to increase the reproducibility. The RCS of mice were derived as described in previous publications (6,10). HcB/Dem (referred to as HcB) strains are derived from the mouse strains C3H/DISnA (referred to as C3H) as background and C57Black/10ScSnA (referred to as C57BL/10) as donor. The CcS/Dem (referred to as CcS) strains are derived form the mouse strains BALB/cHeJ (referred to as BALB/c) as background and STS/A (referred to as STS) as donor as described previously (6,10).

232 Hcb28 F2 hybrid mice were generated by crossing HcB28 to C3H and subsequently intercrossing their F1 progeny. Similarly, 211 Ccs4 F2 hybrid mice were generated by crossing CcS4 and BALB/c mice and intercrossing their F1 progeny. All mice were acclimatized at our animal testing facility for at least one week after transport before the start of the experiments. Mice received a standard laboratory chow (SRM-A, Hope Farms, Woerden, the Netherlands) and tap water ad libitum. All animal experiments were approved by the Institute's Animal Ethics Committee.

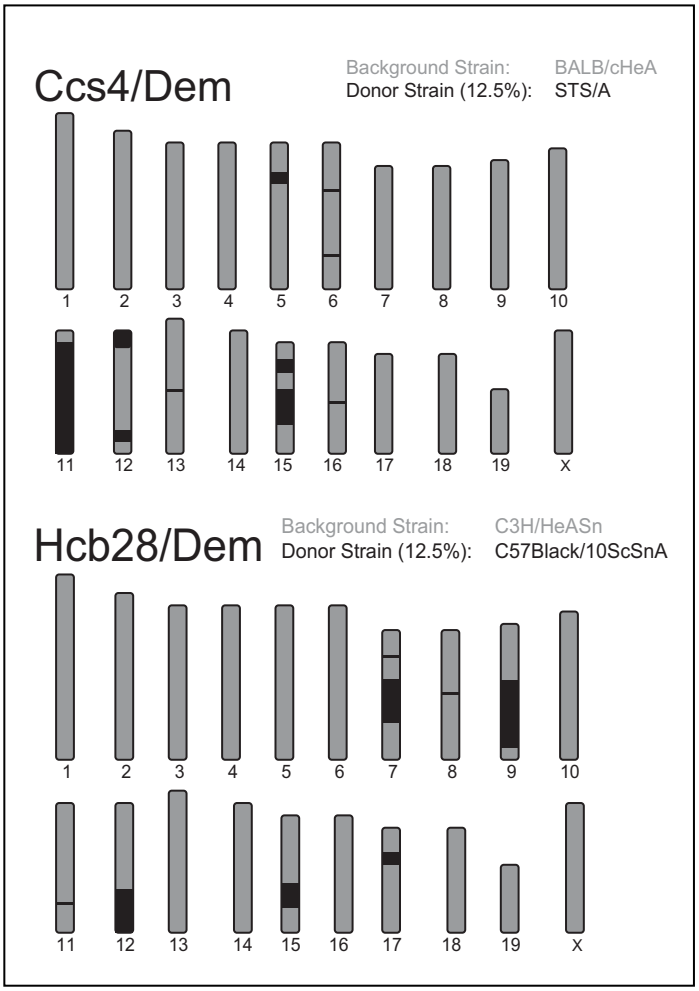

Figure 1: Strain distribution pattern (SDP) of two Recombinant Congenic Strains (RCS). *The CcS4 strain (above) was derived from the mouse strains BALB/c as background (displayed in grey) and STS as donor (displayed in black). The HcB28 (below) was derived by crossings between mouse strains C3H as background (displayed in grey) and C57BL/10 as donor (displayed in black).*

Infection experiments.
In this study, we used the number of viable *B.pertussis* bacteria in the lung one week after inoculation to define the phenotype. This infection protocol was described previously(15,36). Briefly, female mice were intranasally inoculated with 2.10^7 colony forming units (CFU) of *B.pertussis* strain B213 after being anaesthetized with diethyl ether or enflurane. Seven days after inoculation, mice were sacrificed and the lungs were collected in Verwey medium(33). The lungs were homogenized in Verwey medium and diluted 10 and 1,000 times. The number of CFU in these dilutions was determined by plating on Bordet Genou agar substituted with 15% sheep blood and 30μg/ml streptomycin. Plates were incubated for 3 days at 35°C

Genotyping.
Microsatelite markers that have been used to construct the strain distribution patterns (SDP) were also applied to define the genotypes of the F2 hybrid mice. A selection of these SDP's is presented by Jackson Laboratories (24). A more detailed SDP for the HcB series of mice was recently obtained (Demant unpublished work). A schematic representation of SDP's of the two RCS of mice used in this study is presented in Figure 1. In a F2 generation constructed of RCS, only markers present in regions in which the parental strain contains donor genome are informative.
Genomic DNA was isolated from mice tails using the DNeasy Tissue kit (Qiagen). Strain CcS4 carries the genetic material of STS origin on 8 segments on 7 chromosomes as described(11,31). For genotyping, we selected 17 microsatelite markers in these donor regions, D5Mit179, D6Mit109, D11Mit151, D11Mit51, D11Mit139, D11Mit28, D11Mit36, D11Mit122, D11Mit61, D11Mit49, D12Mit37, D15Mit121, D15Mit1, D15Mit3 and D15Mit37. Strain HcB28 carries the genetic material of C57BL/10 origin also on 8 segments on 7 chromosomes (11,11,31). We genotyped these segments using 13 microsatelite markers: D7Mit294, D7Mit350, D7Mit330, D8Rivm46, D9Mit260, D9Mit182, D9Mit82, D11Rivm263, D12Mit167, D12Mit263, D15Mit68, D15Mit107, D17Mit64. Six additional flanking markers were selected round a region of special interest: 3 microsatelite markers, D12Mit53, D12Mit133 and D12Rivm144, and 3 Single Nucleotide Polymorphism (SNP)-markers, S12Rivm101, S12Rivm102, and S12Rivm104. The sequences of all primers except the RIVM-markers (Table 1) were obtained from the mouse genome database of the Massachusetts Institute of Technology (MIT) (26).

Table 1: Markers used for genotyping.

Assay[a]	Forward primer	Reverse primer	C3H	C57Bl/10	Enzyme	NCBI accessnr.
D8Rivm46	CAGCCTGGGCTACATGAGA	TCCTGAGGGAAAATCACACA	164 bp[b]	190 bp		
D11Rivm263	CATGCTAAGCAATGCAGCTC	ACACACAGACCGAGTGACCA	269 bp	252 bp		
D12Rivm144	AGGGAGTCTGAGGCCATCTT	TGCTTCCTTCAGGAACTATTTTT	229 bp	202 bp		
S12Rivm101	GCCATGGGGGAAGATAATTT	CAGAGTGAGGATGGCAGAGG	A	G	SfaN I	rs3685115
S12Rivm102	TGCAGTCGGAGAGAAGGTTT	GCTGGTTCTTCGATCTCCAG	C	T	Tsp45 I	rs3697423
S12Rivm104	GGTGAGAAGTTGGGGACTCA	CCTCCCTTCCTGGATCTCTC	C	G	Bpu10 I	rs4229572

[a] Top 3 markers are microsatelite-markers(MS), bottom 3 are SNP markers.

[b] For MS-markers, fragment length was used as parameter to genotype the F2 back-cross generation.

DNA was amplified in a 10µl PCR-reaction using 5µl hotstar 5x Mastermix (Qiagen), 1.0µM of each primer and approximately 2mM tail-DNA. Amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems), according to the following scheme: an initial 15 minutes at 95°C to denaturize the DNA and to activate the Hotstar Taq, followed by 30 cycles of 45 seconds at 94°C for denaturizing, 45 seconds at 57°C for annealing, 1 minute at 70°C for elongation and finally 10 minutes at 72°C for elongation, PCR-products were stored until further use at 4°C. 6-Carboxyfluorescein (FAM)-labeled microsatelite primer-sets were used (Isogen Life science, Maarssen, the Netherlands) and fragment-sizes were determined on a 3700 Capillary DNA Sequencer/Genotyper system (Applied Biosystems) using Genotyper software (Applied Biosystems). The 3 SNP-markers were analyzed by Restriction Fragment Length Polymorphism (RFLP)-assay on a 2.5% agarose gel (Table 1). Reaction-conditions were used according to the manufacturer's instructions (New England Biolabs).

Statistical analysis.
The statistical differences in phenotypes (number of CFU in the lung) between the different RCS of mice were examined by Analysis of Variance (ANOVA, SPSS) and tested with the Student-Newman-Keuls test for multiple comparisons. To stabilize variances and to obtain approximately normal distributions, the colony forming units were square root (sqrt) transformed. For the F2 mice, linkage between the CFU in the lung and the genotypes, and their effect on the total phenotypic variation, were calculated by an ANOVA with genotypes as fixed factor, and CFU as dependent variable. To correct for the influence of experiment, experiment was included as random factor. All single markers and all pairs of non-linked markers were tested for linkage with a marker or interaction between markers. Interaction, or epistasis, is defined as the combined effect of two or more genes on a phenotype that could not have been predicted as the sum of their separate effects (10). Linkage is presented as p-value and Log of the odds (LOD) score, which was calculated as –log of the significance (p-value).
All markers and interactions were tested at the level of 0.05 ($p<0.05$). p-values were corrected for multiple comparisons using the formula:

$$\mu(T) = [C+2\rho GT^2]\alpha T \quad (18,19)$$

Where μ(T) is the desired corrected p-value, C is the number of chromosomes segregating in the cross (For Hcb28 and Ccs4 C=7, Figure 1), ρ is the crossover rate (1.5 for a F2 hybrid generation), G is the genome length of the segregating part of the donor genome in Morgans (12,5 % of the whole mouse genome of 16M is 2). T^2 is the threshold, the F-value from ANOVA for the observed p-value is used as T^2 , αT is the observed uncorrected p-value.
The estimated effect of a linked locus on the total of the observed phenotypic variation was presented as R^2. To test the reproducibility of the infection protocol, we performed a T-test between the CFU's of the control-mice inoculated in different experiments.
To test a normal distribution of genotypes per experiments, we used a χ^2-test.

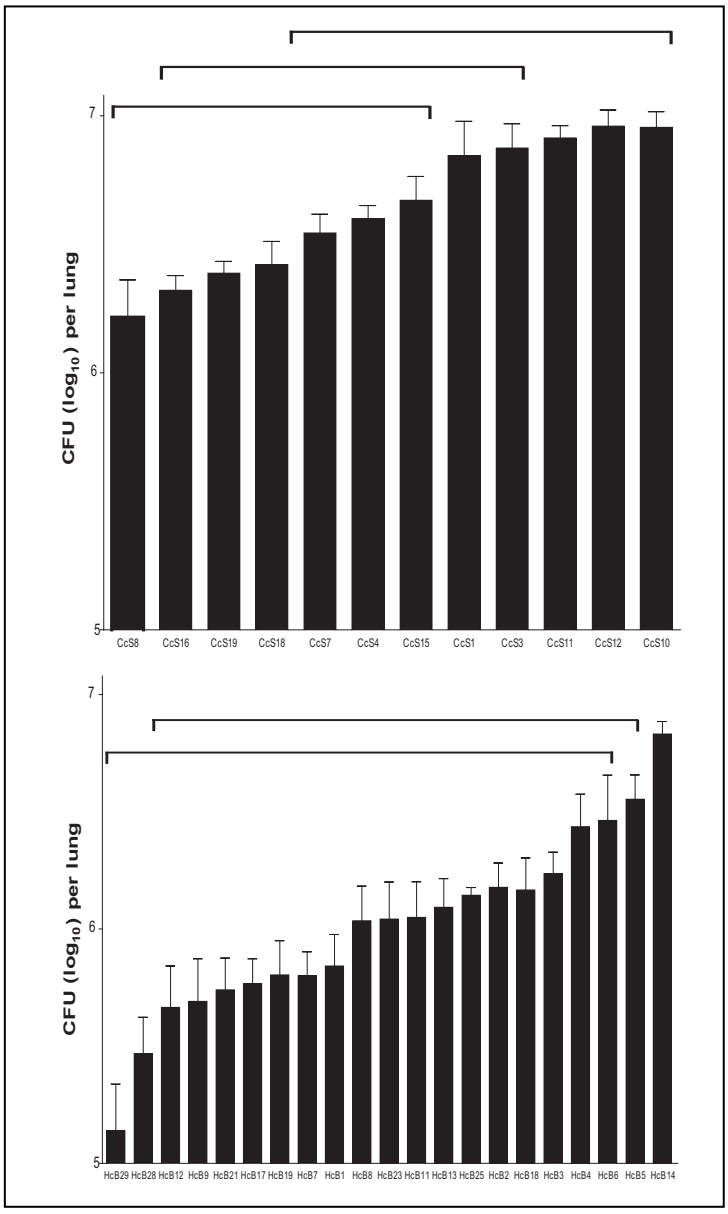

Figure 2. CFU's in the lungs of the CcS Recombinant Congenic Strains (RCS) (Top) and the HcB RCS (Bottom). *Seven days after inoculation with B.pertussis lungs were removed, and the number of viable B.pertussis was determined. Bars indicate the group average of the number of bacteria in the lung (average of 10 mice per group) Horizontal Lines connect groups of mice who are mutually not significantly different according to the Student Neuman Keuls test. From these strains Ccs4 and HcB28 were selected for generating F2 hybrid generations.*

Results

Reproducibility of the infection model

To test the reproducibility of the infection model, we performed several experiments with BALB/c mice. The number of bacteria in the lungs of the control mice, one week post-inoculation, was similar regardless of the day the experiment was performed (data not shown). Because there was no significant difference between any of the control groups ($p>0.05$), we combined the results of all experiments.

Differences between the congenic strains in *B.pertussis* infection of the lung.

We tested twelve different CcS strains for the number of viable bacteria (CFU) in the lung one week post-inoculation. Both in CcS and in HcB mice a wide range in bacterial numbers was found. Results are shown in Figure 2. Significant differences in lung colonization were observed between the strains of mice. Horizontal lines connect groups of mice who are mutually not significantly different according to the Student Neuman Keuls test. The number of CFU varied from $1.6*10^6$ to $9.0*10^6$ CFU per lung (Figure 2). From these strains we selected CcS4 as most representative strain for further breeding.

Similar experiments were performed with twenty-one different HcB strains (Figure 2). The differences in lung colonization in Hcb mice were more pronounced compared to the CcS mice and ranged from $1.4*10^5$ to $6.8*10^6$ CFU per lung. From these strains we selected HcB28 as most resistant strain (HcB29 was not a suitable strain for breeding). We also observed pathologic differences in the lungs, ranging from macroscopically healthy lungs to severe lung edema (data not shown).

Identification of new susceptibility loci

Based on the data presented above, we selected the CcS4 and HcB28 strain for subsequent F2 hybrid experiments to identify susceptibility loci. Both strains were used to generate an F2 hybrid generation of approximately 200 mice. F2 mice were inoculated with *B.pertussis* and the number of CFU in the lung was determined after 7 days (Figure 3). The CFU per lung in the F2 hybrid generation from (CcS4 x BALB/c) ranged from $1.0*10^2$ (detection limit) to $1.8*10^7$, and the average of this group was $3.7*10^6$. The CFU's per lung in the F2 hybrid generation from (Hcb28 x C3H) ranged from $1.0*10^2$ to $3.4*10^6$, and the average of this group was $5.0*10^5$ CFU's per lung. All mice were individually genotyped, and we compared genotypes with phenotypes to identify possible QTL's by linkage analysis.

In the F2 mice, generated by crossing HcB28 and C3H mice, we found linkage between the number of CFU and a region on chromosome 12. In Figure 4 the LOD-diagram is shown for the telomere-region of this chromosome. We found maximum linkage between the number of CFU

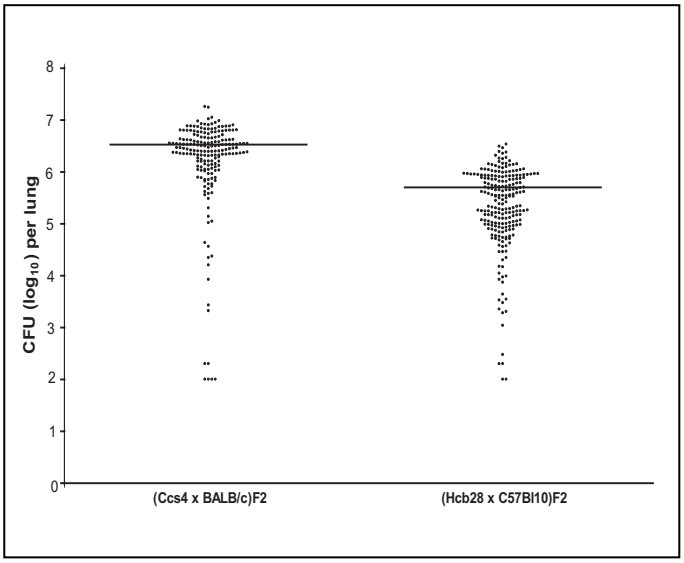

Figure 3: CFU's in the lungs of (CcS4 x BALB/c) F2 and (HcB28 x C57BL/10) F2 mice 7 days after infection with B.pertussis. *Lungs were removed, and the number of viable B.pertussis was determined by counting CFU's in the homogenized lungs. Approximately 200 mice per F2 strain were used. Each dot represents the number of CFU of individual mice.*

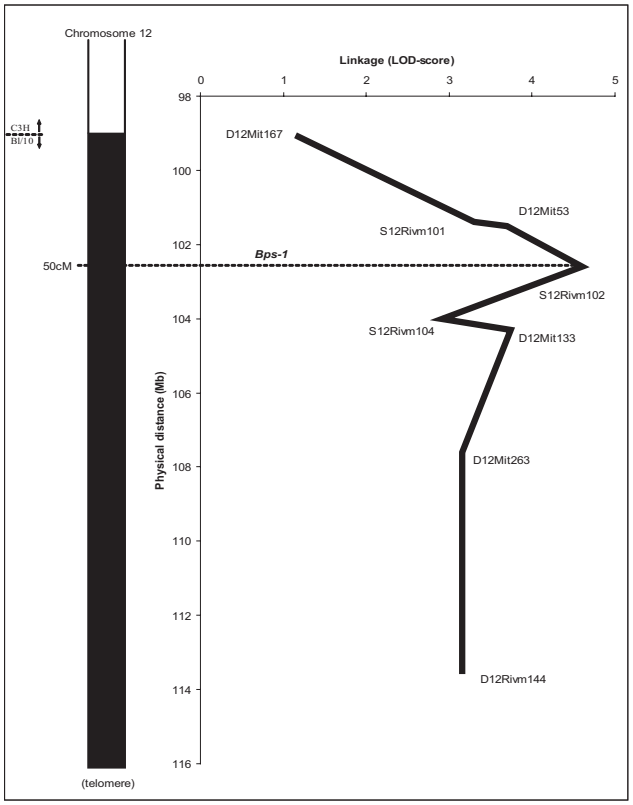

Figure 4: Linkage (LOD-score) between lung colonization by B.pertussis (phenotype) and donor chromosomal loci (genotype). *The association was calculated by ANOVA with genotypes as fixed factor, and sqrt CFU as dependent variable. The LOD score is plotted as -log p against the physical distance of chromosome 12. The region, designated as B.pertussis susceptibilty locus 1 (Bps-1), has a LOD-score of 4,6 (p=0,0000251 , R^2 = 0,09728)*

and the locus defined by S12Rivm102 (102.4Mb) with a LOD-score of 4.6. This LOD-score, and corresponding p-value (p=0.000025) is statistically significant, also after correction for multiple comparisons as described in the statistical section (p=0.0019). However this result does not withstand correction for experiment as random factor (p=0.36). Although the experimental conditions are highly reproducible and the distribution of genotypes per experiment appeared to be normal according to the χ^2-test (p=0.489), the day of the experiment, has influence on the established linkage. We therefore consider these results suggestive linkage instead of significant linkage, and they warrant further investigation. Almost 10 percent (9.7%) of the variation in the number of bacteria in the experiments could be ascribed to the found locus (R^2 = 0.097). We propose to call the locus *B.pertussis susceptibility locus 1 (Bps-1)*. In table 2 means of CFU's in F2 mice are displayed per genotype. The mean differences are significantly different at the 0.05 level between C3H and heterozygotes, and between C3H and C57BL/10, but there is no significant difference between heterozygotes and C57BL/10. This indicates that the presence of C57BL/10 DNA has a dominant positive effect on the clearance of *B.pertussis* from the lung.

Table 2: Linkage between lung colonization by B.pertussis and donor chromosomal loci.

Marker	Position		Experiment not included as factor [a]			Exp. incl. as random factor [b]		Mean CFU [e]			Locus	Strain
	Chromosome	(cM)	p-value	Corrected p [c]	LOD-score [d]	p-value	Corrected p [c]	C3H	Heterozygote	C57Bl/10		
D12Mit167	12	47,0	0,065865	1,549407	1,2	0,129824	2,673846	4,91E+05	3,08E+05	3,83E+05		Hcb28
S12Rivm101	12	47,0	0,000503	0,027409	3,3	0,011481	0,434292	6,26E+05	2,79E+05	3,19E+05		Hcb28
D12Mit53	12	47,0	0,000203	0,012282	3,7	0,002499	0,125222	6,27E+05	2,72E+05	2,61E+05		Hcb28
S12Rivm102 [f]	**12**	**50,0**	**0,000025**	**0,001855**	**4,6**	**0,008272**	**0,359418**	**7,21E+05**	**2,77E+05**	**3,41E+05**	***Bps-1***	**Hcb28**
S12Rivm104	12	52,0	0,001226	0,059666	2,9	0,014378	0,518711	6,20E+05	2,93E+05	3,20E+05		Hcb28
D12Mit133	12	52,5	0,000181	0,011023	3,7	0,033071	0,836288	5,97E+05	2,63E+05	4,14E+05		Hcb28
D12Mit263	12	56,8	0,000705	0,036669	3,2	0,008690	0,365544	6,14E+05	2,92E+05	3,42E+05		Hcb28
D12Rivm144	12	56,8	0,000705	0,036669	3,2	0,008690	0,365544	4,01E+05	3,10E+05	2,39E+05		Hcb28
D5Mit179	5	4,4			Not significant [g]			2,68E+06	2,70E+06	2,79E+06	***Bps-2***	Ccs4
D11Mit122	11	50,3			Not significant [g]			2,76E+06	2,68E+06	2,78E+06	***Bps-3***	Ccs4
D5Mit179 x D11Mit122 [g]			**0,010250**	**0,281342**	**2,660718**	**0,002184**	**0,089410**	***Please see table 3***				**Ccs4**

[a] The association was calculated by ANOVA with genotype(s) as fixed factor and sqrt CFU as dependent variable.
[b] The association was calculated as above but with experiment included as random factor.
[c] p-value corrected for multiple comparisons according to Lander and Kruglyak (18)
[d] LOD-score was presented as –log (p)
[e] Means of CFU's in F2 mice per genotype.
[f] Loci with highest linkage are presented in bold.
[g] Individual markers showed no significant linkage, but linkage was found between the number of CFU's and the interacting markers D5Mit179 and D11Mit122.

Table 3: Genetic interaction between D5Mit179 and D11Mit122[a].

		D11Mit122		
		BALB/c	Heterozygote	STS
D5Mit179	BALB/c	3,98E+06	2,31E+06	2,28E+06
	Heterozygote	2,95E+06	2,32E+06	3,36E+06
	STS	1,35E+06	3,67E+06	2,22E+06

[a] Means of CFU's in F2 mice per genotype of two interacting markers.

In the F2 mice generated by crossing CcS4 with BALB/c, we did not find any linkage between individual markers and the number of CFU's. However, we found linkage between the number of CFU's and the interacting markers D5Mit179 (chromosome 5) and D11Mit122 (chromosome 11) with a LOD-score of 2.92 (Figure 5). This LOD-score, and corresponding p-value (p=0.010), are statistically significant, but not after correction for multiple comparisons as described in the statistical section (p=0.28). However when the experiment is included as random factor the significance increases (p=0.089). We therefore consider these results suggestive linkage, instead of significant linkage, and they warrant further investigation.
Seven percent (7.0%) of the variation in the number of bacteria in the experiments could be ascribed to these interacting loci (R^2 = 0.070). We propose to call these loci *Bps-2* and *Bps-3*. In table 3 means of CFU's in F2 mice are displayed per interaction of D5Mit179 and D11Mit122 (the marker-combination with the highest linkage) genotypes. When both markers carry the BALB/c allele in stead of STS, the CFU's in the lung is relatively high.

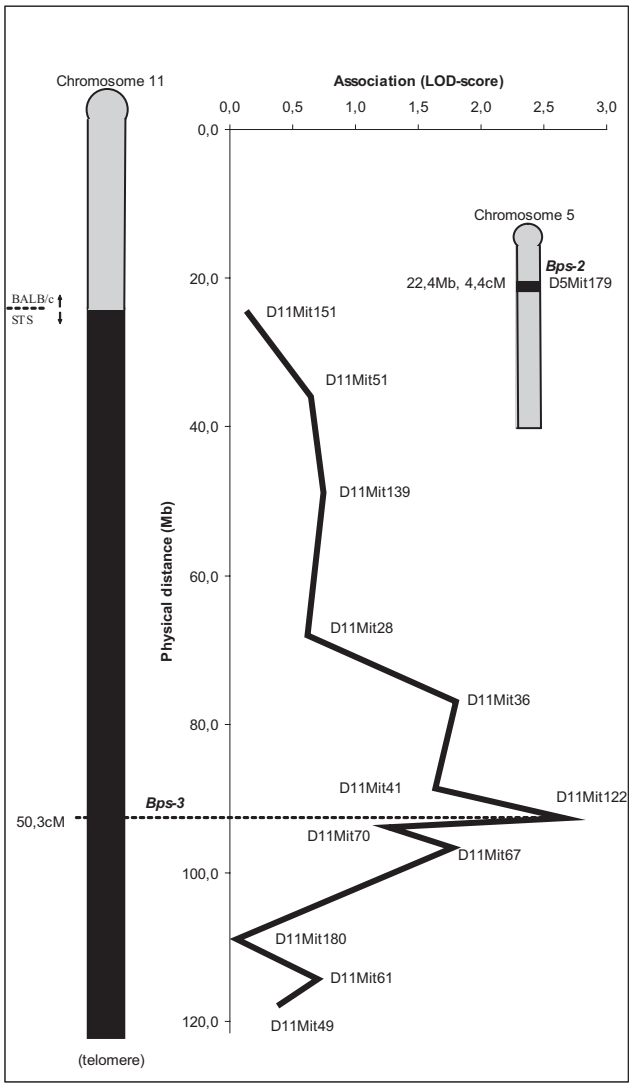

Figure 5: Linkage (LOD-score) between lung colonization by B.pertussis (phenotype) and an interaction between two donor chromosomal loci (genotype). *The association was calculated by ANOVA with the interaction of 2 genotypes as fixed factor, experiment as random factor and sqrt CFU as dependent variable. Individual markers showed no linkage with the phenotype, but an interaction was found between chromosome 11 and an locus on chromosome 5 (4.4cM). The LOD score is plotted as -log p against the physical distance of chromosome 11. These regions designated as B.pertussis susceptibilty locus 2(chromosome 5) and 3 (chromosome 11) (Bps-2, Bps-3), has a LOD-score of 2.7 (p=0,002184 , R^2 = 0,070)*

Discussion

Pertussis is re-emerging, despite high vaccination coverage's (27). Further, pertussis morbidity and mortality is especially high in baby's that are too young to be vaccinated (32). Treatment of pertussis with antibiotics is only effective if initiated early during infection. Thus new novel therapeutic or preventive treatments of pertussis are required.

Elucidation of genetic differences in susceptibility to complex diseases can result in improved knowledge of pathways involved in the pathogenesis of these diseases. Identification of genes which affect susceptibility to infectious diseases is important to unravel pathogen-host interactions. Furthermore, identification of such genes may lead to novel therapeutics. Novel therapeutics are especially important for infectious diseases which are difficult to control by vaccination such as tuberculosis, pertussis and Aids, and/or antibiotics such as multi-resistant bacteria.

To identify genes that affect the course of *B.pertussis* infection we tested 2 sets of recombinant congenic strains, STS and HcB sets. This approach has been applied successfully before in identifying *Sst1* as one of the loci in mice affecting susceptibility to *Mycobacterium tuberculosis* (16,17). The same approach was also successful in identifying a series of loci (*Lmr*), each associated with a different combination of pathological and immunological reactions, to *Leishmania major* (1,20,34).

In this study we studied the effect of genetic differences in the mouse on lung colonization by *B.pertussis.* Recently a mutation in the Toll Like Receptor 4 (*Tlr-4*) was identified as a major factor that influences the course of *B.pertussis* infection in mice (13,22). The gene coding for Tlr4 is positioned on mouse chromosome 4 (33cM). The strains of mice we applied here do not have documented mutations in Tlr4, and this gene therefore did not influence the variation observed in our experiments.

We tested two sets of RCS of mice, and selected one strain of each set for generating F2 hybrid generations to identify susceptibility loci. For both sets of RCS, a wide range was observed in the number of CFU recovered from the lungs. The phenotypic variation in these strains of mice, which only differ in 12.5% of their genome, indicates that genetic factors do influence *B.pertussis* pathogenesis. In addition, the range of variation between these strains suggests that the course of pertussis is controlled by multiple genes, and that the genetics of *B.pertussis* infection is complex and controlled by multiple QTL's.

To identify loci potentially responsible for the observed differences in lung colonization, we generated F2 hybrid generations of approximately 200 mice. As shown in Figure 3, the phenotypic differences of the F2 mice generated from the CcS4 were approximately similar compared to the F2 generated from the HcB28. Based on the SDP's we genotyped all mice from both F2 hybrids, and compared the genotypes with the degree of lung colonization. For a classical mouse F2 intercross generation(whole genome segregating) the following critical values

for establishing linkage are used: a LOD-score below 2.8 argues against linkage, while a LOD-score of 4.3 or higher indicates linkage (18,19). For a RCS F2 hybrid generation, the critical values are lower because only 12.5% of the genome is segregating. Therefore all p-values are corrected for multiple comparisons as described in the statistical analysis section.

In the F2 mice generated from the HcB28 mice, we initially found linkage between lung colonization and one marker D12Mit133 (chromosome 12) with a LOD-score of 2.7. We subsequently tested several flanking markers and found a maximum linkage between CFU and S12Rivm102 with a LOD-score of 4.6. This finding could explain almost 10% of the variation in CFU observed in the F2 mice generated from the HcB28 (Figure 4). This locus, which we propose to designate as *B.pertussis susceptibility locus 1 (Bps-1*), is positioned near the telomere on chromosome 12 (approximately 102Mb), and influences the number of bacteria present in the lung one week after inoculation. As is shown in Table 2, the presence of C57BL/10 DNA on this allele in stead of C3H DNA has a dominant positive effect on the clearance of bacteria from the lung.

In the F2 mice generated by crossing CcS4 with BALB/c, we did not find any linkage between individual markers and the number of CFU's. However, we found linkage between the number of CFU and the interacting markers D5Mit179 (chromosome 5) and D11Mit122 (chromosome 11) with a LOD-score of 2.92 (Figure 5). Seven percent (7.0%) of the variation in the number of bacteria in this experiment could be ascribed to these interacting loci (R^2 = 0.070). We propose to call these loci *Bps-2* and *Bps-3*. In table 3 means of CFU's in F2 mice are displayed per interaction of D5Mit179 and D11Mit122 (the marker-combination with the highest linkage) genotypes. These linked regions are homologous to the human loci 7q21-q22 (D5Mit179) and 17p11-q12 (D11Mit122). These regions contain numerous genes, including genes encoding with immunological functions, but the regions must be narrowed down before focusing on individual genes is possible.

The significance of the results described may not withstand all statistical corrections, but nevertheless the results are promising enough for future investigation. We consider the 3 novel loci to be in suggestive linkage. Because, according to the χ^2-test, the genotypes on the *Bps-1* locus are normally distributed (p=0.489) the relevance of the *Bps-1* locus is underlined.

Because the wide range of variation found in the RCS mice suggests a multigenic control of *B.pertussis* infection, we did not expect to find only three loci influencing our phenotype. It is therefore likely that the combined effect of other loci on this phenotype is too subtle to detect in these experiments. Presumably, because *B.pertussis* infection is controlled by multiple genes, the extremes in phenotypes must be larger to identify subtle genetic effects. Although all mice were raised and housed in the same facility under carefully controlled conditions, the contribution of other factors, besides genetics, can not be excluded completely as complicating factors in the differences in bacterial load between

mice. In addition it would probably be necessary to increase the number of F2 animals to detect significant QTLs.
Based on the data presented in Figure 4, we selected the region between 100 and 108Mb around *Bps-1* on chromosome 12 for future analysis. This region is similar to the human locus 14q32. It contains 84 different genes, including genes involved in immunity such as tumor necrosis factor-associated factor 3, and microtubule affinity-regulating kinase 3. However the majority of genes in this region has an unknown function (RIKEN cDNA). In future experiments we aim to identify the gene(s) that modifies the susceptibility to *B.pertussis* by a combination of positional cloning and expression analysis.
In conclusion, we screened two sets of RCS of mice for susceptibility to *B.pertussis* infection, and found a wide range in bacterial numbers in the lung at one week post inoculation. This indicates a multigenic control of the *B.pertussis* infection. We identified one locus located on chromosome 12, which we designated as *Bps-1,* and 2 interacting loci on chromosome 5 and 11, designated *Bps-2* and *3,* which influence the number of bacteria in the lung one week after inoculation. The presence of C57BL/10 DNA on *Bps-1* in stead of C3H DNA has a dominant positive effect on the clearance of bacteria from the lung.

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Chapter 3

Lung response to *Bordetella pertussis* infection in mice identified by gene-expression profiling.

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Abstract

Host genetics determines the course of *Bordetella pertussis* infection in mice. Previously we found four loci, Tlr4 and three novel loci, designated *Bps 1-3,* that are involved in the control of *B. pertussis* infection. The purpose of the present study was to identify candidate genes that could explain genetic differences in the course of *B. pertussis* infection assuming that such genes are differentially regulated upon infection. We therefore studied the course of mRNA expression in the lungs after *B. pertussis* infection. Of the 22,000 genes investigated, 1,841 were significantly differentially expressed, with 1,182 genes up- and 659 genes down-regulated. Up-regulated genes were involved in immune-related processes, such as the acute phase response, antigen presentation, cytokine production, inflammation, and apoptosis, while down-regulated genes were mainly involved in non-immune processes, such as development and muscle contraction. Pathway analysis revealed the involvement of granulocyte function, Toll-like receptor signaling pathway, and apoptosis. Nine of the differentially expressed genes were located in *Bps-1,* 13 were located in *Bps-2* and 62 were located in *Bps-3.* We conclude that *B. pertussis* infection induces a wide and complex response, which appears to be partly specific for *B. pertussis* and partly non-specific. We envisage that these data will be helpful in identifying polymorphic genes that affect the susceptibility and course of *B. pertussis* infection in humans.

Introduction

The airway pathogen *Bordetella pertussis* can cause the disease known as whooping cough. Despite high vaccination coverage, pertussis is still an endemic disease with peaks in incidence every two to three years during the last decade (10-12). Susceptibility to and severity of *B. pertussis* infection in infants and children vary widely (22). The spectrum of clinical symptoms ranges from subclinical infection to mild disease, severe whooping cough, and death. In previous studies we examined genetic susceptibility of mice to *B. pertussis*, and identified loci in the mouse genome that are involved in restriction of colonization and / or clearance of *B. pertussis*. We and others have provided indications for the role of several polymorphic host genes in the course of *B. pertussis* infection, including the interferon gamma receptor (27), toll-like receptor 4 (Tlr4) (15,28,29), and three putative disease loci, *B. pertussis susceptibility locus 1, 2 and 3 (Bps-1, 2 and 3)* (3). *Tlr4* is a major host factor involved in the course of *B. pertussis* infection. C3H/HeJ mice, which cary a non functional *Tlr4* gene, exhibit an aberant course of infection. Functional *Tlr4* is essential for an efficient IL1-β, TNF-α and IFN-γ response, efficient clearance of bacteria from the lung, and therewith reduced lung pathology (4). The *Bps-1* locus is located on chromosome 12, spanning a region of 185 genes and has a dominant positive effect on the clearance of *B. pertussis* from the lung. The function of most genes in this locus is unknown. Two other loci, *Bps-2* and *Bps-3,* showed genetic interaction and are located on chromosomes 5 and 11, respectively (3).

The highest linkage between the number of CFU and the *Bps-1* locus that we observed showed a LOD score of 4.6. This LOD score and the corresponding *P* value (P = 0.000025) are statistically significant, also after correction for multiple comparisons (P = 0.0019). We interpreted these data as suggestive for linkage warranting further investigation (3).In the present study we aimed to enhance the insight in the host response and pathogenesis of *B. pertussis* infection by identifying genes that are differentially expressed upon infection. In particular we were interested in identifying candidate genes that could explain genetic differences in the course of *B.* pertussis infection, assuming that such genes are differentially regulated upon infection. For this purpose we infected mice and analyzed gene-expression profiles in the lungs on days one, three and five after inoculation using microarrays. We were especially interested if genes, located in the *Bps-1, 2* and *3* loci, were differentially expressed after *B. pertussis* infection or that the susceptibility of these loci was regulated alternately.

Materials and Methods

Experimental design

Female C3H/DISnA mice (eight mice per group, total 48) were intranasally inoculated with $2*10^7$ colony forming units (CFU) of the streptomycin-resistant Tohama strain of *B. pertussis* (B213) in 40 µl Verwey medium (The Netherlands Vaccine institute, Bilthoven, the Netherlands), or as a control with Verwey Medium only. One, three and five days after inoculation, mice were euthanized. To remove blood cells from the lungs, mice were perfused with PBS, and the lungs and trachea were collected (23,44). For the extraction of RNA, the right lung was collected in RNA stabilization reagent (RNAlater, Qiagen, Venlo, The Netherlands). The left lung was fixed intratracheally using 4% formalin for histological examination.

The number of viable *B. pertussis* bacteria was determined in the trachea to confirm a proper infection (42). Therefore, approximately one centimeter of the trachea was collected in 500 µl Verwey medium. Bacterial suspensions were diluted in Verwey medium and the number of CFU was determined by plating on Bordet Gengou agar supplemented with 15% sheep blood and 30 µg/ml streptomycin (Tritium Microbiology, Veldhoven, the Netherlands). Plates were incubated for 4 days at 35°C before counting the number of CFU's using a ProtoCOL Colony counter (Synbiosis, Cambridge, United Kingdom.).

Animals

Female C3H/DISnA mice were kindly supplied by Dr. Peter Demant (Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York). Mice were bred to the appropriate numbers at our animal testing facility in Bilthoven, The Netherlands. Mice were acclimatized for at least one week before the start of the experiments. Mice received standard laboratory food (SRM-A, Hope Farms, Woerden, the Netherlands) and tap water ad libitum. All animal experiments were approved by the Institute's Animal Ethics Committee and were performed according to NIH guidelines (32) .

Clinical and pathological examinations

Mice were weighed before inoculation, and subsequently every day after inoculation to determine the relative change in weight. Lung weights were determined post mortem as a parameter for lung inflammation. Formalin-fixed lungs were embedded in paraplast (Monoject Inc., St Louis, Missouri). Sections (5 µm) were stained with hematoxylin-eosin. Lung lesions were examined for infiltration of inflammatory cells in the peribronchiolar space (peribronchiolitis), infiltration of inflammatory cells in the alveoli (alveolitis), infiltration of inflammatory cells in the perivascular space (perivasculitis), hypertrophy of mucus-producing glands, free protein (exudate), and eosinophilia. Lung lesions were scored semi-quantitatively as absent, minimal, slight, moderate, marked, or strong, as previously described (5).

Transcription profiling

Lungs were incubated in RNAlater (Qiagen, Venlo, The Netherlands) at 4°C for one day, after which the tissues were transferred to a fresh tube, and stored at -80°C. RNA was extracted by using the midi-RNA isolation kit (Qiagen, Venlo, The Netherlands). RNA concentrations were measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). RNA quality was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California), and was expressed as the RNA Integrity Number (RIN) as defined by the manufacturer. Only samples with a RIN score of at least eight (on a 1-10 scale) were used for expression profiling.

Microarray slides were spotted at the Microarray Department of the University of Amsterdam. The slides contain 21,997 65-mer oligo nucleotides from the Sigma-Compugen Mouse oligonucleotide library, 192 additional 65-mer oligo nucleotides of genes of which 185 were located in the *Bps-1* region spotted in duplicate (Isogen Life science, IJsselstein, the Netherlands), as well as appropriate controls and blank spots.

Based on RNA quality and yield, RNA isolated from six mice per group was selected for amplification and labeling. RNA amplification and labeling was carried out using the Amino Allyl MessageAmp II aRNA kit (Ambion Inc., Austin, Texas), using 1 µg of total RNA as starting material. RNA samples from individual mice were labeled with Cy3. A common reference containing a RNA pool of all samples isolated was labeled with Cy5. After hybridization and washing, the arrays were scanned using a ScanArray 4000XL microarray scanner (Perkin-Elmer, Wellesley, Massachusetts).

Median Cy3 and Cy5 spot signals were determined using ArrayVision (Imaging Research, St. Catharines, Ontario, Canada). Quality control was performed by means of visual inspection of the scanned images, raw data scatter, and MA plots (M is a mnemonic for the log-ratios of expression, A is a mnemonic for the log-intensity of each spot) (39), as well as a normal probability plot to assess signal distribution (34). If more than 10% of the spots were flagged as missing data, slides were excluded from data-analysis. At least 5 slides per group were used for further analysis.

Raw microarray signal data were normalized in the R statistical software environment (37), using a four-step approach of (1) natural log-transformation, (2) quantile normalization of all scans, (3) correcting for differences in the reference signal, and (4) averaging replicate spot data.

Significance of differences in gene-expression between the experimental groups was calculated in R with ANOVA. The false discovery rate (FDR) was calculated according to Benjamini and Hochberg (8). The FDR is the expected proportion of false positives among the genes considered significantly differentially expressed (7,8). Gene-expression data were visualized by hierarchical clustering (using Euclidian distance and Ward linkage) using GeneMaths (Applied Maths, St-Martens-Latem, Belgium). For this purpose, gene-expression values were

corrected for the overall average to allow a neutral comparison between all *B. pertussis*- and mock-inoculated groups. Gene categories were defined by Gene Ontology (GO, http://www.geneontology.org). Classification and enrichment according to GO categories were determined using DAVID/EASE (13,16), using EASE-scores as *P*-value for enrichment. Additional pathway analysis was performed using MetaCore™ (GeneGo Inc., http://www.genego.com).

Results

Bacteriologic and pathologic findings

All *B. pertussis*-inoculated mice had between 10^2 and 10^5 CFU's in the trachea during the first five days after inoculation thereby confirming a proper infection. A statistically significant decrease in the number of bacteria in the trachea was observed over time during the first five days post inoculation (P=0.022, linear regression, SPSS, data not shown).

Infected mice lost up to four percent of their body weight during the first five days post inoculation, while all mock-inoculated mice gained weight up to four percent during these five days. This difference in relative gain of weight between mock- and *B. pertussis*-inoculated mice is significant from day one till day five post inoculation (Figure 1).

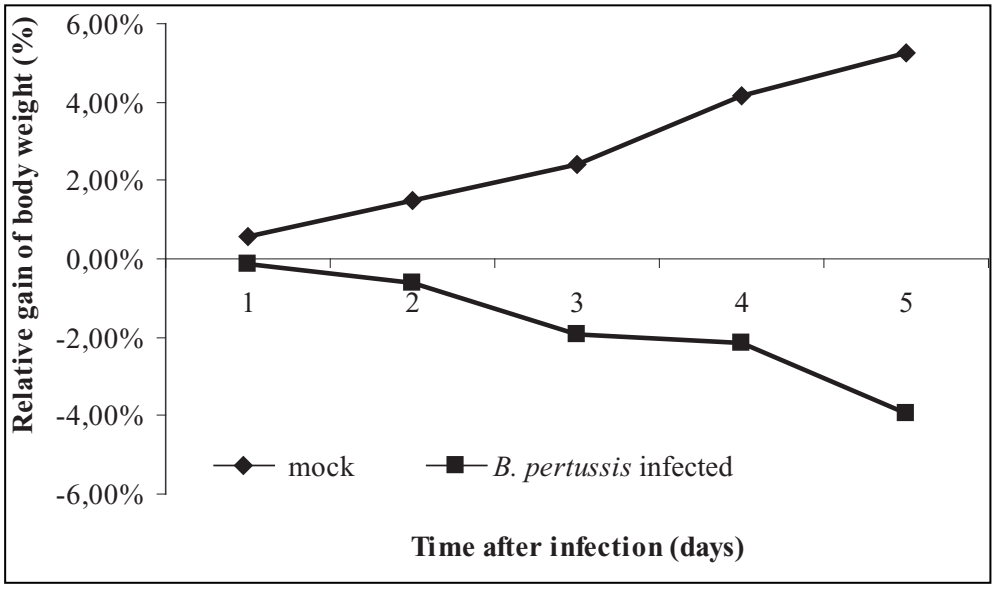

Figure 1: gain of body weight after infection
Relative gain of body weight at different days post inoculation with medium (mock) or B. pertussis. Boxes represent the mean of the relative weights of B. pertussis infected mice, diamonds represent the mean of the relative weights of mock inoculated mice.

We determined lung weights in relation to body weight, as a marker for inflammation. All mock-inoculated mice had relative lung weights of approximately one percent during the first five days after inoculation, while *B. pertussis*-infected mice had relative lung weights of up to three percent of their total body weight. This difference in relative lung weights between mock- and *B. pertussis*-inoculated mice is significant from day one till day five post inoculation (Figure 2).

A summary of histopathological findings is presented in Table 1. Intranasal inoculation of *B. pertussis* causes an acute inflammatory response that is characterized by influx of polymorphonuclear leukocytes (PMNs) and macrophages, starting in perivascular and peribronchiolar areas on day one, and extending to alveolar walls and lumina on days three and five.

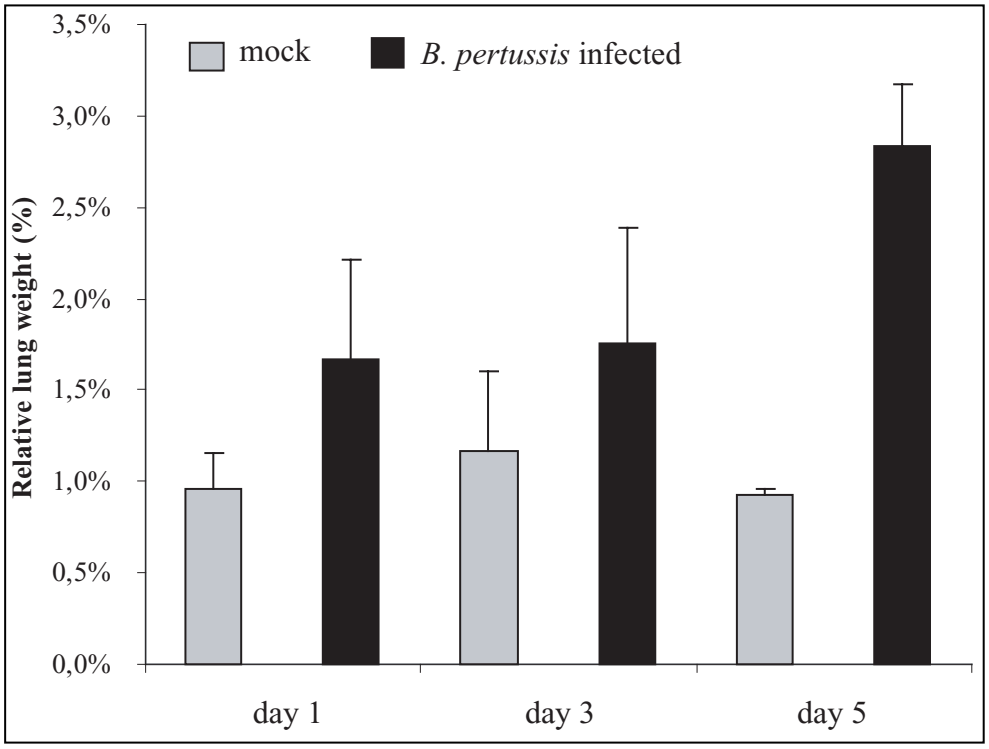

Figure 2: Lung weight after infection
Relative lung weight of mice after infection with B. pertussis at several days post inoculation. Solid bars represent the mean relative weight of B. pertussis infected mice, grey bars represent the mean relative lung weight of mock inoculated mice.

Table 1: Histological evaluation of lung changes after B. pertussis inoculation.

Parameter and grade		Number of Animals [a]					
		Mock			*B.pertussis*		
		Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Peribrochiolitis [b]	Minimal	2		3	4	2	2
	Slight				1	2	5
	Moderate					3	
	Total [c]	(25%)	(0%)	(38%)	(63%)	(88%)	(88%)
Perivasculitis	Minimal	2	2	2	2	1	
	Slight				4	3	3
	Moderate					3	5
	Marked					1	
	Total	(25%)	(25%)	(25%)	(75%)	(100%)	(100%)
Hypertrophy	Minimal	1		1	1	2	
	Slight						1
	Moderate					3	1
	Marked					2	4
	Strong						1
	Total	(13%)	(0%)	(13%)	(13%)	(88%)	(88%)
Alveolitis	Minimal				2	1	
	Slight				4		2
	Moderate					3	1
	Marked					1	3
	Strong					3	1
	Total	(0%)	(0%)	(0%)	(75%)	(100%)	(88%)
Exudate	Minimal					2	2
	Slight					2	2
	Moderate					1	1
	Marked					1	
	Strong					1	
	Total	(0%)	(0%)	(0%)	(0%)	(100%)	(63%)

[a] *Eight mice per group were inoculated with medium (mock) or B. pertussis*

[b] *Hematoxylin-eosin-stained slides were examined for peribronchiolitis, alveolitis, perivasculitis, hypertrophy of mucus-producing glands and free protein(exudate). Lung lesions were scored semi-quantitatively as absent (not included in table), minimal, slight, moderate, marked, or strong*

[c] *Total percentage of animals with at least minimal scoring in the specified category*

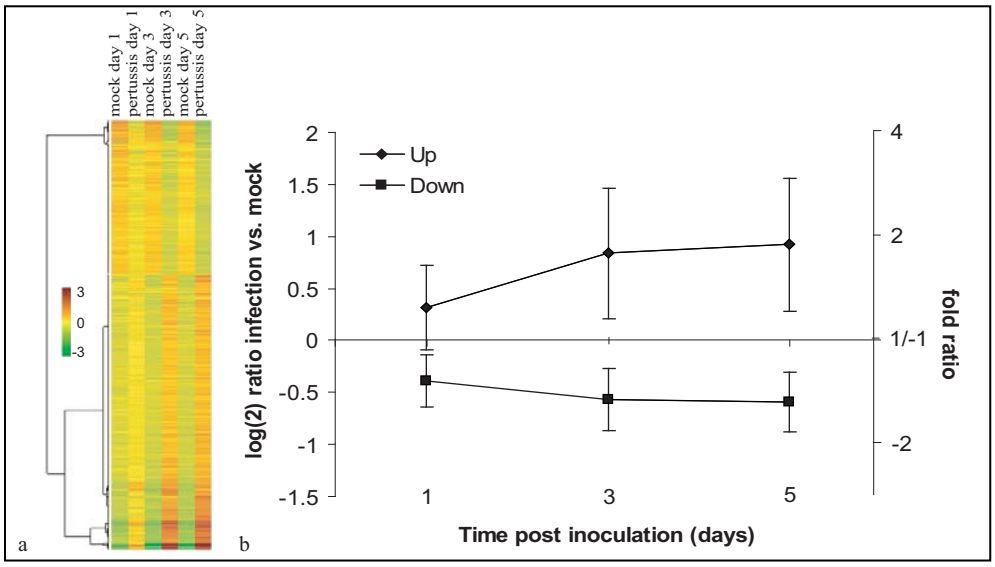

Figure 3: representation of microarray data from mock- and B. pertussis-inoculated mice. *Analysis was performed on 1,841 genes whose expression was found to be significant at an FDR < 0.05 (a) Hierarchical clustering shows the segmentation of the genes clustered by gene-expression. Relatively increased gene-expression is presented in red while relatively decreased gene-expression is presented in green. The color scale shows log(2) ratios of gene-expression levels compared to the global average.*
(b) Course of B. pertussis regulated gene-expression, diamonds represent the average of 1,182 up-regulated genes and squares represent the average of 659 down-regulated genes. Error bars show the standard deviation (SD) of the average gene-expression.

Expression profiling

Gene-expression profiles were determined in at least five animals per group. The three mock-inoculated groups, euthanized at days one, three and five, were statistically indistinguishable (i.e. there were no genes with FDR < 0.6). Of the approximately 22,000 genes tested, 1,841 genes, were significantly differentially expressed in *B. pertussis*-inoculated lungs (FDR <0.05, corresponding to P<0.0042). Expression levels of all 1,841 genes as well as comparisons to other studies are presented in the supplementary data Table 5.

Hierarchical clustering showed time-dependent changes in gene-expression with 1,182 up-regulated genes and 659 down-regulated genes. Of these genes, 439 were more than two-fold up-regulated and 134 were more than two-fold repressed. All genes followed a similar trend in time. One day post inoculation differential gene-expression was already observed, but the effect was larger on days three and five post inoculation (Figure 3). There was no identifiable subset of genes that displayed a different kinetic trend in gene-expression.

To assign functions to differentially expressed genes, we classified them according to Gene Ontology (GO) categories. In addition, we determined enrichment for GO-categories to identify pathophysiological processes involved

in the response to *B. pertussis*. The most important GO categories, i.e. with the highest percentage differentially expressed genes, are summarized in Table 2. Most up-regulated genes are involved in immune-response-related processes, such as the acute phase response, antigen presentation, cytokine activity, inflammation and apoptosis, whereas down-regulated genes are involved in non-immune processes, such as development, muscle-contraction, cytoskeleton and general processes like metabolism.

Table 2: Classification of genes induced upon B. pertussis infection[a]

	Up		Down[b]		Total on array	*P*-value[c]
Acute-phase response[d]	12	(55%)	0	(0%)	22	$2.4*10^{-7}$ (up)
Antigen presentation	21	(66%)	0	(0%)	32	$7.7*10^{-15}$ (up)
Apoptosis	47	(15%)	6	(2%)	324	$8.0*10^{-5}$ (up)
Cell cycle	43	(8%)	22	(4%)	510	$4.3*10^{-1}$ (up)
Chemokine Activity	21	(58%)	0	(0%)	36	$1.2*10^{-13}$ (up)
Complement activation	8	(26%)	1	(3%)	31	$9.7*10^{-3}$ (up)
Cytokine Activity	48	(25%)	6	(3%)	192	$7.8*10^{-13}$ (up)
Cytoskeleton	31	(6%)	40	(8%)	531	$2.0*10^{-3}$ (down)
Development	96	(7%)	94	(7%)	1433	$9.8*10^{-1}$ (down)
Immune response	162	(40%)	3	(1%)	408	$6.3*10^{-75}$ (up)
Inflammatory response	46	(38%)	2	(2%)	121	$1.1*10^{-19}$ (up)
Metabolism	353	(7%)	220	(5%)	4771	$1.8*10^{-1}$ (down)
Muscle contraction	0	(0%)	12	(24%)	49	$6.7*10^{-6}$ (down)
Listed in any of the above	576	(9%)	318	(5%)	6574	
Other	328	(6%)	204	(4%)	5639	
Unannotated	278	(3%)	137	(1%)	9744	
Total	1182	(5%)	659	(3%)	21957	

[a]Classification according to Gene-Ontology (GO) categories
[b]Analysis was performed on 1841 regulated genes
[c] Ease Score for GO category enrichment in the specified set of genes
[d]Note:some genes are classified into multiple categories.

To reveal inter-gene relationships, we performed MetaCore™ analysis on the 1,841 regulated genes in addition to the GO-based analysis presented above. The most significant pathways are presented in Table 3. This analysis revealed the significance of several pathways, such as regulation of granulocyte function, the Toll-like receptor (TLR) signaling pathway, the classic complement pathway and apoptosis.

Genes located in *Bps-1* that were differentially regulated upon *B. pertussis* inoculation and their function according to the GO database are presented in Table 4. *Bps-1* contains 185 genes, of which nine were differentially expressed. The strongest up-regulated genes in this locus were tryptophanyl-tRNA synthetase (*Wars,* 4-fold), and tumor necrosis factor alpha-induced protein 2 (*Tnfaip2,* 5.4-fold). Zinc finger FYVE domain containing 21 (*Zfyve21,* 1.5-fold) and kinesin family member 26A (*Kif26a,* 1.7-fold) were the strongest down-regulated genes.

Genes located in *Bps-2* and *-3* that were differentially regulated upon *B. pertussis* inoculation are presented in Supplementary data Table 6. *Bps-2* is located on chromosome 5 and contains 262 genes of which 13 were differentially

expressed. Interleukin-6 (IL-6) was the strongest up-regulated gene (4.7-fold) in this locus and general transcription factor IIIC, polypeptide 2, beta was the strongest down-regulated gene (2-fold) in this locus. *Bps-3* is located on chromosome 11 and contains 922 genes of which 62 were differentially expressed. Chemokine (C-C motif) ligand 8 was the strongest up-regulated gene (15.3-fold) in this locus and RIKEN cDNA 9530033F24 gene was the strongest down-regulated gene (2.3-fold) in this locus.

Table 3: Pathway Analysis of genes induced upon B. pertussis infection[a]

Name of Pathway involved[b]	Main process[c]	*P*-Value[d]	Number of genes in Pathway		
			Expressed		Total
Transcription regulation of granulocyte development	Immune response	0.000001925	17	(38%)	45
TLR ligands and common TLR signalling pathway	Immune response	0.00001814	16	(34%)	47
ECM remodeling	Metabolism	0.00003906	18	(30%)	60
Role of IAP-proteins in apoptosis	Apoptosis	0.00005458	13	(36%)	36
HETE and HPETE diosynthesis and metabolism	Metabolism	0.0003249	13	(31%)	42
TPO signaling via JAK-STAT pathway	Cell cycle	0.0003893	9	(39%)	23
Leukotriene 4 biosynthesis and metabolism	Metabolism	0.0004655	16	(27%)	60
RhoA regulation pathway (extension, GEFs/GAPs)	Immune response	0.002412	10	(29%)	34
MIF in innate immunity response	Immune response	0.002435	14	(25%)	57
Retinol metabolism	Metabolism	0.002842	12	(26%)	46
Classic complement pathway	Complement activation	0.002842	12	(26%)	46
Putative Erythropoietin Signaling Pathway (part 1)	Immune response	0.002905	13	(25%)	52
MAPK cascade	Cell cycle	0.004975	12	(24%)	49
Ceramide-dependent NO antiapoptotic action	Apoptosis	0.006738	13	(23%)	57
IL9 signaling pathway	Cytokine activity	0.006809	9	(27%)	33
Ephrins signaling	Other	0.007847	13	(22%)	58
Polyamine metabolism	Metabolism	0.008731	7	(30%)	23
Integrin outside-in signaling	Cell cycle	0.009444	16	(20%)	79
Chemokines and adhesion	Chemokine activity	0.009657	34	(16%)	209

Definition of abbreviations: Extracellular matrix (ECM), GTPase-accelerating protein (GAP), guanine nucleotide exchange factor (GEF),
hydroperoxyeicosatetraenoic acid (HPETE), hydroxyeicosatetraenoic acid (HETE), inhibitor of apoptosis (IAP), interleukin 9 (IL9),
Janus tyrosine kinase (JAK), macrophage migration inhibitory factor (MIF), mitogen-activated protein kinase (MAPK), nitric oxide (NO),
ras homolog gene family, member A (RhoA), signal transducers and activators of transcription (STAT)
[a]Analysis was performed on 1841 genes whose expression was found to be significant at an FDR < 0.05, The list is presented by descending p-value.
[b]Classification according to main Gene-Ontology (GO) categories
[c] P-Value represent the probability of particular mapping arising by chance, given the numbers of genes in all Metacore pathways,
genes involved in the specified pathway and genes regulated upon B. pertussis inoculation.

Table 4: Genes located in Bps-1 that are differentially regulated upon B. pertussis inoculation

GenBank accession	Gene Symbol	Location [a]	Fold Ratio change			*P*-value	Description	Pathway[b]
			day 1	day 3	day 5			
NM_011705	Vrk1	106	1,29	1,52	1,41	0,00003	vaccinia related kinase 1	protein kinase activity
NM_011710	Wars	109	1,95	3,58	4,01	0,00000	tryptophanyl-tRNA synthetase	aminoacyl-tRNA ligase activity
NM_010480	Hspca	111	-1,14	1,40	1,27	0,00064	heat shock protein 1, alpha	protein folding
NM_009396	Tnfaip2	112	1,43	4,48	5,40	0,00000	tumor necrosis factor, alpha-induced protein 2	angiogenesis
XM_138272	Gm266	112	-1,08	-1,25	-1,35	0,00110	gene model 266, (NCBI)	G-protein coupled receptor activity
NM_026752	Zfyve21	112	-1,33	-1,41	-1,50	0,00001	zinc finger, FYVE domain containing 21	zinc ion binding
XM_138275	Kif26a	113	-1,32	-1,68	-1,73	0,00030	kinesin family member 26A	microtubule motor activity
NM_178911	Pld4	113	1,21	1,82	2,52	0,00000	phospholipase D family, member 4	catalytic activity
XM_619046	LOC434166	113	1,13	1,42	1,42	0,00141	similar to hypothetical protein MGC37588	unknown

[a] Bps-1 is located on chromosome 12, location is presented as distance from centromere (Mb)
[b]Classification according to Gene-Ontology (GO) categories

Discussion

We have recently shown that genetic differences of the host play an important role in the restriction of colonization and /or clearance of *B. pertussis* from the lungs of mice (3,4). In the present study we wanted to extend the understanding of the host response to *B. pertussis*, and identify genes and pathways that play a role in the pathophysiological response to *B. pertussis* infection. In particular we were interested in identifying candidate genes that could explain genetic differences in the course of *B.* pertussis infection, assuming that such genes are differentially regulated upon infection. For these purposes we studied the time course of mRNA-expression in the lungs of mice after a *B. pertussis* infection by microarray analysis. We were especially interested if genes, located in the *Bps-1, 2* and *3* loci, were differentially expressed after *B. pertussis* infection or that the susceptibility conferred by these loci was regulated alternately.

Of the approximately 22,000 genes investigated, we found 1,841 (8.4%) genes to be significantly differentially expressed. Differential gene-expression was already observed on day one post inoculation, but the maximum effect was observed on day three post inoculation. This effect remained constant till at least day five post inoculation. The observed course in gene-expression after *B. pertussis* inoculation was similar to that induced by another gram-negative lung pathogen, *Francisella tularensis* (2). In a study of expression profiles in the lungs of *F. tularensis*-infected C57BL/6 mice, 424 (2.3%) genes out of the 18,500 genes investigated were up-regulated. Genes involved in immune responses were activated strongly on day four post inoculation, but hardly or not at all on days one and two (2).

As expected, most up-regulated genes are involved in immune and inflammation-related processes, such as antigen presentation, complement, cytokine and chemokine activity. Other processes involved include the acute phase response and metabolism. Most down-regulated genes are involved in non-immune processes, such as metabolism, development, cell-cycle or muscle contraction. We speculate that by down-regulating these genes, the energy balance is shifted in favor of the immune response. Evidently, cellular influx in the lungs is likely to influence the gene-expression profile.

Our data provide more detailed information on the central role of PMN's in the pathogenesis of *B. pertussis* infection. Pathogenesis of *B. pertussis* infection is characterized by colonization and proliferation of the ciliated mucosal cells, resulting in damage of the respiratory epithelium, and an acute increase in the levels of inflammatory cytokines resulting in cellular infiltrate in the alveolar spaces (30,31,35). Circulating PMN's are rapidly recruited to the lungs to bind and ingest *B. pertussis*, subsequently killing the bacteria by a combination of reactive oxygen and granule components. Finally, the PMN's undergo apoptosis (25). TLR ligands such as LPS are critical components for the recruitment and priming of PMN's. Serum antibody–mediated clearance of *B. pertussis* also

requires a TLR-induced early recruitment of PMN's. However, pertussis toxin limits this rapid serum antibody–mediated clearance by inhibiting PMN recruitment (24). As determined by pathway analysis in this study, the most significant pathways involved in the response to *B. pertussis* infection are regulation of PMN function, TLR signaling pathway, extracellular matrix remodeling, and apoptosis. It has been described that apoptosis of PMNs in which TLR2 plays an important role (18), is accelerated following phagocytosis of bacteria (25,26). So, the involvement of three out of four pathways may, at least partly, be explained by the influx of PMNs. Our data thus further illustrate the central role of PMN's in the course of *B. pertussis* infection and the involved mechanisms in PMN function.

We found the response to *B. pertussis* to be wide and complex. Some remarkable findings were the involvement of mucin genes, Intracellular pathogen resistance gene, and the excessive up-regulation of Serum amyloid A genes. We found the mucin (Muc) genes *Muc4* and *Muc5b* to be significantly upregulated. Expression of mucin in the respiratory tract is one important innate host defense mechanism that respiratory pathogens must overcome (6). *Muc4,* which encodes a respiratory tract mucin glycoprotein, was expressed 3.3-fold at day five upon *B. pertussis* inoculation. *Muc5b,* which encodes mucin 5, subtype B protein, in tracheobronchial epithelial cells, was expressed 1.8-fold three days upon inoculation. Belcher et al. (6) describe that *B. pertussis* induces mucin gene transcription by BEAS-2B cells. Evidently gene-expression studies do not allow to define the functional role of regulated genes. Further studies should define whether the mucin response is favorable to the host, the pathogen, or both.

In our study we found a 3-fold upregulation of the Intracellular pathogen resistance gene 1 (*Ipr1*). *Ipr1* mediates innate immunity to *Mycobacterium tuberculosis* (33). Pan et al. (33) speculated that the *Ipr1* gene product might function in integrating signals generated by intracellular pathogens with mechanisms controlling innate immunity, cell death, and pathogenesis. Our data therefore suggest that *Ipr1* may be involved in intracellular presence of *B. pertussis*. Interestingly, *B. pertussis* was shown to survive intracellularly in macrophages, PMN's and broncho-alveolar lavage (BAL) cells of mice (14,31).

Among the upregulated genes upon *B. pertussis* inoculation, Serum amyloid A (SAA) genes were upregulated to a remarkably high extent. SAA is an acute phase protein whose expression is markedly up-regulated during inflammation and infection (40). Among other functions, this protein plays an important role in HDL cholesterol metabolism by promoting cellular cholesterol efflux through a number of different efflux pathways (41). In our study, SAA1, 2 and 3 were induced 14-, 54- and 44-fold respectively after *B. pertussis* infection, again underlining the important role for the acute phase response after *B. pertussis* infection.

Although strongly upregulated, it is clear that the acute-phase response is not specific for *B. pertussis* infection.
In a previous study we observed a 1.5 fold up-regulation of the membrane-receptor *Tlr4* and a 5-fold up-regulation of the downstream cytokine gene *Tnf-α* three days post *B. pertussis* inoculation by means of quantitative real-time PCR. Thus, a slight upregulation (1.5-fold) of this receptor induces a more pronounced (5-fold) induction of this downstream pro-inflammatory cytokine (4). In the present study we obtained similar results by using microarray analysis. *Tlr4* expression was increased 1.6-fold, while the expression of *Tnfaip* was increased 4.5-fold 3 days post inoculation. The results of the present study hereby confirm our previous observations. In a recent study a high correlation between gene-expression values determined by quantitative real-time PCR and microarray was observed (9). Given these results and our observations, we conclude that the results, at least for the two given genes, are similar for both techniques.
It is very likely that the expression profile to *B. pertussis* is partly unique to *B. pertussis,* and partly non-specific. To examine the specificity of *B. pertussis*-regulated gene-expression, we compared our data to other studies. In a recent review by Jenner & Young (19), a comparison was made between published transcriptional profiling data from 32 studies that involved 77 different host-pathogen interactions. A set of 417 genes that comprise a common host transcriptional response was defined. We found that 122 genes affected by *B. pertussis* infection were listed in this common host pathogen response, further illustrating that many genes regulated by *B. pertussis* are indeed not specific for *B. pertussis* infection, but are involved in a common response to pathogens. One explanation for the broad response in expression profiles is the complexity of the bacterium. It is, for instance, known that lipopolysaccharide (LPS) is a very potent immune stimulus, which induces a strong inflammatory response (21,36,43). Transcriptional profiling of LPS-induced acute lung injury in mice resulted in 71 immune-related up-regulated genes (20). 43 of these 71 genes were also regulated upon *B. pertussis* inoculation in this study.
Previously we have demonstrated that the *Bps-1* locus has a dominant positive effect on the clearance of *B. pertussis* from the lung. Of the 185 genes in this locus, nine were differentially expressed. Some of these genes could explain the linkage of this locus with susceptibility to *B. pertussis* infection. Tumor necrosis factor, alpha-induced protein 2 (*Tnfaip2)* has been described as a tumor necrosis factor-alpha-inducible primary response gene in endothelial cells (38). Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNA with their cognate amino acid. Tryptophanyl-tRNA synthetase (*Wars*) catalyzes the aminoacylation of tRNA(trp) with tryptophan and is induced by interferon (17).The role of the nine genes in the *B. pertussis* infection process is unknown, and will be investigated in future experiments. Thus, by using a microarray approach, we selected nine possible candidate genes from the 185 present in

Bps-1. However, by this approach, we focussed on transcriptionally regulated genes, and the possibility remains that susceptibility to *B. pertussis* is regulated in a different way, such as phosphorylation or protein binding.
In comparison to the *Bps*-1 region, the *Bps-2* and *-3* regions contain considerably more genes, namely 262 and 922 genes, respectively, of which 13 and 62 were differentially expressed post *B. pertussis* inoculation. Among these differentially expressed genes are several interesting candidate genes that may explain the linkage of these loci with susceptibility to *B. pertussis* infection. IL-6 was the most strongly induced gene in the *Bps-2* region (4.7-fold). IL-6 is a pro-inflammatory cytokine that can be secreted by macrophages after Tlr4 activation (1). Among the genes differentially expressed in the *Bps-3* region was a large group of chemokines of which Chemokine (C-C motif) ligand 8 was the strongest up-regulated gene (15.3-fold). Upon *B. pertussis* infection this large group of chemokines is induced to promote immune cells to migrate to the site of infection. The role of the other *B. pertussis*-induced genes in these loci is still unknown, and will be investigated in future experiments.
In conclusion, we found 1,841 genes in the lungs of mice that are differentially expressed after *B. pertussis* inoculation. Most up-regulated genes are involved in immune and inflammation related processes or in non-specific processes. Most down-regulated genes are involved in non-immune processes. A substantial number of genes and pathways provide more detailed information on the central role of PMN's in the pathogenesis of *B. pertussis.* In particular the transcriptional profiles indicate the significance of genes involved in TLR activation, the recruitment and activation of PMN's, and apoptosis.

Supplementary data

Raw data as well as the detailed description of the experiment will be uploaded to the freely accessible online database ArrayExpress http://www.ebi.ac.uk/arrayexpress/ upon acceptance of the manuscript. Expression levels of all 1,841 genes as well as comparisons to other studies are presented in the supplementary data Table 5.

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Chapter 4

Comparative gene expression profiling in two congenic mouse strains following *Bordetella pertussis* infection.

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Abstract
Susceptibility to *Bordetella pertussis* infection varies widely. These differences can partly be explained by genetic host factors. HcB-28 mice are more resistant to *B. pertussis* infection than C3H mice, which could partially be ascribed to the *B*. *pertussis susceptibility locus-1* (*Bps1*) on chromosome 12*.* The presence of C57BL/10 genome on this locus instead of C3H genome resulted in a decreased number of bacteria in the lung.
To further elucidate the role of host genetic factors, in particular in the *Bps1* locus, in *B. pertussis* infection, and to identify candidate genes within in this region, we compared expression profiles in the lungs of the C3H and HcB-28 mouse strains following *B. pertussis* inoculation. Twelve and a half percent of the genomes of these mice are from a different genetic background.
Upon *B. pertussis* inoculation 2,353 genes were differentially expressed in the lungs of both mouse strains. Two hundred and six genes were differentially expressed between the two mouse strains, but, remarkably, none of these were up- or down-regulated upon *B. pertussis* infection. Of these 206 genes, 17 were located in the *Bps1* region. Eight of these genes, which showed a strong difference in gene expression between the two mouse strains, map to the immunoglobulin heavy chain complex (*Igh*).
Gene expression changes upon *B. pertussis* infection are highly identical between the two mouse strains despite the differences in the course of *B. pertussis* infection. Because the genes that were differentially regulated between the mouse strains only showed differences in expression before infection, it appears likely that such intrinsic differences in gene regulation are involved in determining differences in susceptibility to *B. pertussis* infection. Alternatively, such genetic differences in susceptibility may be explained by genes that are not differentially regulated between these two mouse strains. Genes in the *Igh* complex, among which *Igh-1a/b*, are likely candidates to explain differences in susceptibility to *B. pertussis.* Thus, by microarray analysis we significantly reduced the number of candidate susceptibility genes within the *Bps1* locus. Further work should establish the role of the *Igh* complex in *B. pertussis* infection.

Background

Bordetella pertussis is a gram-negative bacterium that can cause the respiratory disease known as pertussis or whooping cough in humans. Susceptibility to this disease and its course vary widely between individuals (7). We have previously shown that genetically divergent mouse strains differ in their response to *B. pertussis* infection, underlining that infection is influenced by host genetic factors(3,4). In addition, a role of several host genetic loci in the course of *B. pertussis* infection has been indicated, such as the toll-like receptor 4 (Tlr4) gene (3,4,17,18), the interferon gamma receptor gene (16), and three novel loci, *B. pertussis susceptibility locus 1, 2, and 3 (Bps1, 2, and 3)* (3) that showed linkage with the severity of infection.

We have used recombinant congenic mouse strains (RCS) as a tool to facilitate the mapping of low-penetrance quantitative trait loci that control complex traits such as a *B. pertussis* infection (8). RCS are derived from two different inbred strains, the so-called background and donor strain. After two backcrosses and subsequent brother-sister mating, a set of RCS is created, with each strain containing 12.5% of the donor genome differently distributed across the background genome (9). HcB/Dem RCS of mice are derived from two backcrosses of the inbred mouse strains C3H/DISnA (C3H) as background and C57BL/10ScSnA (B10) as donor strain, resulting in 12.5% B10 genome across the C3H genome. The genome of each HcB/Dem strain, thus, differs maximally 12.5% compared to the background strain (C3H) (Figure 1a) (12,31).

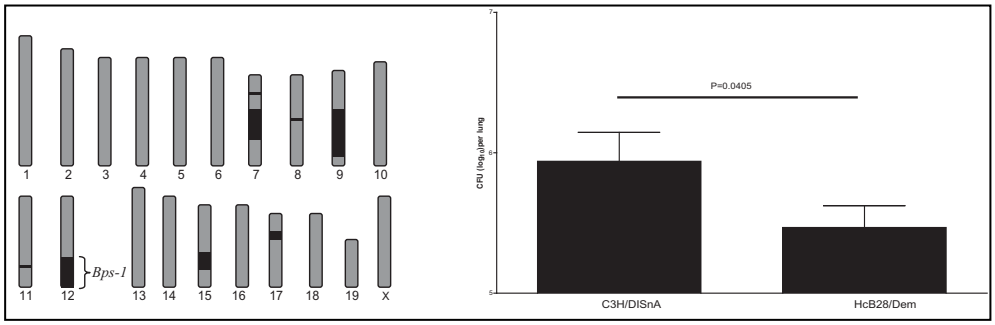

Figure 1: Differences between C3H/DISnA and HcB-28/Dem mice.
a) Illustration of the distribution pattern of B10 genome across the background genome of the C3H strain of the Recombinant Congenic Strain HcB-28/Dem. The HcB-28 strain was derived by crossings between mouse strains C3H/DISnA as background (displayed in grey) and C57BL/10 as donor (displayed in black). b) Mice (C3H/DISnA) were infected intranasally with B. pertussis strain B213. The lungs were removed seven days after inoculation, and the number of viable B. pertussis was determined. Bars represent the average number of bacteria in the lungs. Horizontal line indicates the significant difference between groups. Error bars indicate the Standard Deviation (SD).

HcB-28 mice contained lower numbers of bacteria in their lungs seven days post-inoculation compared to C3H mice. Subsequent genotyping led to the identification of the three susceptibility loci, *Bps1, 2,* and *3.* Especially *Bps1* displayed strong linkage with susceptibility to *B. pertussis* infection. The *Bps1* locus is located on chromosome 12, spanning a region of 185 genes, of which one or more genes have a dominant positive effect on the clearance of *B. pertussis* in the lung, and/or the reduction of bacterial multiplication. However, the mechanism by which genes within this region influence the course of infection is not clear. Two other loci, *Bps-2* and *Bps-3,* showed genetic interaction and are located on chromosomes 5 and 11, respectively (3).
Although *Bps-1* has not yet been validated, we believe that the significance of this locus warrants further study. To further elucidate the role of host genetic factors, in particular the *Bps1* locus, in *B. pertussis* infection, and to identify candidate genes within this region, we studied expression profiles in the lungs of mice following *B. pertussis* inoculation. The traditional approach for mapping genes in susceptibility loci is a combination of positional cloning and linkage analysis (26,28). Although this strategy has proven to be effective (8,27), the approach is quite costly and animal-consuming. Previously we identified changes in gene expression in the lungs of C3H/DISnA mice after *B. pertussis* infection, and we especially focused on differentially expressed genes in the lungs of infected and non-infected mice located in *Bps1,-2,* and *-3* (2). We found that the expression of 1,841 genes was significantly changed upon *B. pertussis* infection. These genes are involved in various immune-related processes, such as the acute-phase response, antigen presentation, cytokine production, inflammation, and apoptosis. Nine of the differentially expressed genes are located in *Bps1,* 13 are located in *Bps-2,* and 62 are located in *Bps-3.*
In the present study we compared the gene expression profiles in the lungs of two mouse strains, i.e. HcB-28/Dem and C3H, which showed a different course of *B. pertussis* infection, in order to further identify candidate susceptibility genes without the need for positional cloning. We hypothesized that the phenotypic differences displayed by these mice in the response to *B. pertussis* can partly be explained by a different gene expression profile between the mouse strains, and that this approach could lead to the identification of candidate genes affecting the course of infection. Using this approach we reduced the number of candidate susceptibility genes within the *Bps1* locus.

Methods

Experimental design

Forty-eight female HcB-28/Dem and 48 female C3H/DISnA (the background strain of the HcB-28/Dem) mice were intranasally inoculated with $2*10^7$ colony forming units (CFU) of the streptomycin-resistant Tohama strain of *B. pertussis* (B213) in 40 µl Verwey medium (The Netherlands Vaccine Institute, Bilthoven, the Netherlands), or as a control with Verwey Medium only (total of 96 mice). One, three, and five days after inoculation, 8 infected and 8 control mice were euthanized. To remove blood from the lungs, mice were perfused with phosphate-buffered saline (PBS, Tritium Microbiology, Veldhoven, the Netherlands). Subsequently, the lungs and trachea were collected (14,35). For RNA extraction, the right lung was collected in RNA stabilization reagent (RNAlater, Qiagen, Venlo, the Netherlands). For histological examination, the left lung was fixed intratracheally using 4% formalin.

The number of viable *B. pertussis* bacteria was determined in the trachea to confirm a proper infection (33) (Note that tracheal counts are not representative for bacterial clearance from the lungs.) To this end, approximately one centimeter of the trachea was collected in 500 µl Verwey medium. Bacterial suspensions were diluted in Verwey medium and the number of CFU was determined by plating on Bordet Gengou agar supplemented with 15% sheep blood and 30 µg/ml streptomycin (Tritium Microbiology). Plates were incubated for 4 days at 35°C and the resulting colonies were counted using a ProtoCOL Colony counter (Synbiosis, Cambridge, United Kingdom).

All *B. pertussis*-infected mice had between 10^2 and 10^5 CFU in the trachea during the first five days after inoculation, confirming an actual infection (data not shown).

For the analysis of intrinsic immunological and cellular differences between the two mouse strains, independent from infection, blood and spleen samples were collected from five untreated female mice of both strains.

Animals
Breeding pairs of C3H/DISnA and HcB-28/Dem were supplied by the Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York. Female mice were bred to the appropriate numbers at our animal testing facility in Bilthoven, the Netherlands. Mice were acclimatized for at least one week before the start of the experiments. Mice received standard laboratory food (SRM-A, Hope Farms, Woerden, the Netherlands) and tap water ad libitum. All animal experiments were approved by the Institute's Animal Ethics Committee and were performed according to NIH guidelines (21) and Dutch legislation.

Clinical and pathological examinations
Mice were weighed before inoculation, and subsequently every day after inoculation to determine the relative change in weight. Lung weights were determined post mortem as quantitative parameter for lung inflammation. Formalin-fixed lungs were embedded in paraplast (Monoject Inc., St Louis, MO). Sections (5 µm) were stained with hematoxylin-eosin. Lung lesions were examined for infiltration of inflammatory cells in the peribronchiolar space (peribronchiolitis), infiltration of inflammatory cells in the alveoli (alveolitis), infiltration of inflammatory cells in the perivascular space (perivasculitis), hypertrophy of mucus-producing glands, free protein (exudate), and eosinophilia. Lung lesions were scored semi-quantitatively as absent, minimal, slight, moderate, marked, or strong, as previously described (5).

Enumeration of cellular subsets
Spleens were harvested and weighed. Cell suspensions were prepared and the number of nucleated cells per spleen was determined using a Coulter Counter Z2 (Beckman Coulter, Mijdrecht, the Netherlands). The percentage of B- and T-cells, as well as T-cell subsets were determined using a fluorescence-activated cell sorter (FACS-Calibur, BD Biosciences, Alphen aan den Rijn, the Netherlands). T-cells were detected with phycoerythrin-labeled (PE) anti-CD3ε antibodies (Molecular probes, Invitrogen, Breda, the Netherlands). CD4+ T-cells were determined using allophycocyanin-labeled (APC) antibodies (Molecular probes) and CD8+T-cells were determined using fluorescein isothiocyanate-labeled (FITC) antibodies (Molecular probes). B-cells were determined using PE-labeled anti-CD19 antibodies (Molecular probes).

Splenocyte proliferation test
The cell suspensions were used to measure the spontaneous (medium only) or stimulus-induced ^{3}H-thymidine uptake. The cells were stimulated by adding 10 µg/ml Concavalin A (Con A, T-cell stimulus), 70 µg/ml lipopolysacharide (LPS, B-cell stimulus) or 15 µg/ml Lectin (B- and T-cell stimulus) to the culture medium.

Cells were incubated for 72hr at 37°C. ^{3}H-thymidine was present during the final 24hr of culture. See (34) for details.

Hematology
The morphologic hemogram was determined using an ADVIA 120 Hematology system (Multispecies analyzer, Bayer, Mijdrecht, the Netherlands).

Immunoglobulin quantification
Blood was collected in Vacuette Minicollect tubes (Greiner bio-one, Alphen a/d Rijn, the Netherlands), and serum was removed by centrifugation. Immunoglobulins were determined using the multiplex Beadlyte Mouse Immunoglobulin Isotyping Kit (Millipore, Billerica, MA) for the Luminex platform (Luminex, Oosterhoud, the Netherlands), as prescribed by the manufacturer.

Transcription profiling

Microarray analysis experiments were performed as described previously (2). Briefly, total RNA was extracted from lungs and amplified using the Amino Allyl MessageAmp II aRNA kit (Ambion Inc., Austin, TX). RNA amplification was performed to obtain more nucleic acid for labeling. This results in a stronger fluorescence signal and a better signal/noise ratio. Because of this, less experimental samples (and therefore animals) are needed to obtain sufficient statistical power. RNA samples from individual mice were labeled with Cy3. A common reference containing a RNA pool of all samples isolated was labeled with Cy5.

Microarray slides were spotted at the Microarray Department of the University of Amsterdam. The slides contain 21,997 65-mer oligonucleotides from the Sigma-Compugen Mouse oligonucleotide library, 192 additional 65-mer oligonucleotides of genes of which 185 are located in the *Bps1* region spotted in duplicate (Isogen Life science, IJsselstein, the Netherlands), as well as appropriate controls and blank spots. The *Bps-1* oligonucleotides were annotated according to NCBI mouse genome build 34.1; all other oligonucleotides were annotated according to the Sigma-Compugen Mouse oligonucleotide library.

At least 5 slides per group were used. Raw microarray signal data were normalized and analyzed in the R statistical software environment (24,25,29). Significance of differences in gene expression between the experimental groups was calculated in R with ANOVA. The false discovery rate (FDR) was calculated according to Benjamini and Hochberg (6). Gene expression data were visualized by hierarchical clustering (using Euclidian distance and Ward linkage) using GeneMaths (Applied Maths, St-Martens-Latem, Belgium). Gene categories were defined by Gene Ontology (GO, http://www.geneontology.org). Classification and enrichment according to GO categories or location were determined using DAVID/EASE (10,13), using EASE-scores as *P*-value for enrichment.

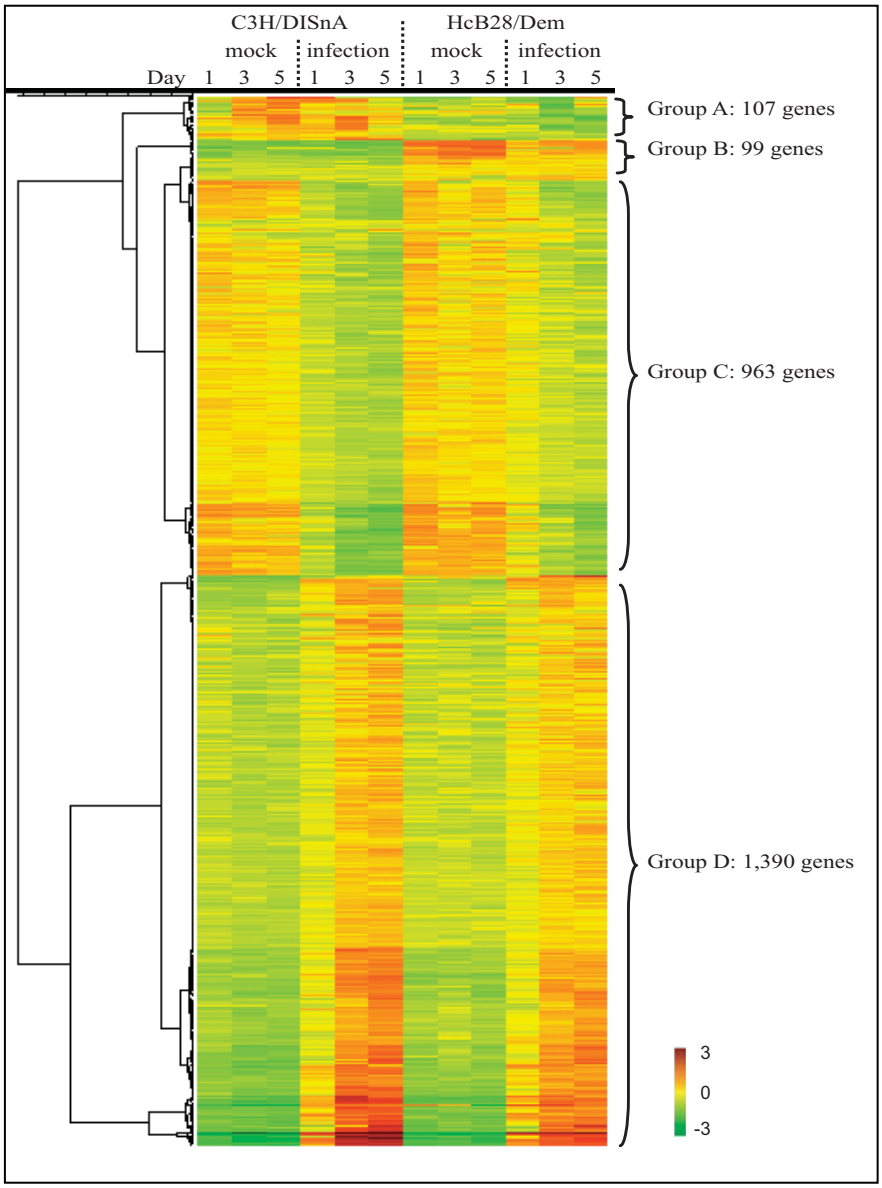

Figure 2: Presentation of microarray data from mock- and B. pertussis-inoculated mice. *Analysis was performed on 2,559 genes whose expression was found to be significantly up- or down-regulated at an FDR < 0.05. Relatively increased gene-expression is presented in red while relatively decreased gene-expression is presented in green. The color scale shows log(2) ratios of gene-expression levels compared to the global average. ANOVA analysis identified 206 genes that were differentially expressed between the two mouse strains and 2,353 genes that were differentially expressed after infection. Hierarchical clustering of the 2,559 regulated genes results in four distinct groups, higher in C3H (A), higher in HcB-28 (B), down-regulated after infection in both strains (C) and up-regulated after infection in both strains (D).*

Results

Response of C3H and HcB mice to infection

We have previously examined clearance of *B. pertussis* from the lungs of infected RCS mice, and established that HcB-28 mice contained lower numbers of bacteria in their lungs seven days post inoculation compared to C3H mice (Figure 1b) (3). To examine whether the genetic differences between "resistant" HcB-28 mice and "susceptible" C3H mice also result in a different transcriptional response to *B. pertussis* infection, we compared gene expression profiles of the lungs of infected and uninfected HcB-28 and C3H mice (2,3). mRNA transcription profiles were determined in at least five animals per group.

The three mock-inoculated groups of each mouse strain, euthanized at days one, three and five, were found to be statistically indistinguishable from each other, i.e. both within the same strain and between strains (i.e. there were no genes with FDR < 0.6). Of the approximately 22k genes tested, 2,559 genes were significantly differentially expressed either in the two mouse strains or upon *B. pertussis*-inoculation (FDR <0.05, corresponding to P<0.0058). The gene expression levels of all 2,559 genes are presented in the supplementary data Table 3. The expression profiles of all genes are illustrated by hierarchical clustering in Figure 2. ANOVA analysis identified 206 genes (groups A and B) that were differentially expressed between the two mouse strains before inoculation, and 2,353 genes (groups C and D) that were differentially expressed in both strains after infection. Remarkably, there were no genes that were differentially expressed between the two mouse strains following inoculation. Thus, genes up- or down-regulated upon infection were identically regulated in both mouse strains despite their clear genetic differences and differences in *B. pertussis* susceptibility.

The 206 genes that were differentially expressed between the two strains were all unaffected by infection, but displayed a difference in basal expression level. Of the 206 strain-dependent genes, 107 genes were expressed at a higher level in C3H/DISnA mice (group A) and 99 genes were expressed at a higher level in the HcB-28/Dem mice (group B). Of the 2,353 infection-regulated genes, 963 were down-regulated upon infection (group C), and 1,390 were up-regulated upon infection (group D). All genes that were affected by *B. pertussis* infection followed a similar trend in time. One day post-inoculation differential gene expression was already observed, but the effect was stronger on days three and five post-inoculation. Thus there was no identifiable subset of genes that displayed a different kinetic trend in gene expression.

To assign functions to the differentially expressed genes, we classified them according to Gene Ontology (GO) categories. In addition, we determined enrichment for location and biological functions to identify pathophysiological processes involved in the response to *B. pertussis* inoculation. The most important GO categories, i.e. with the highest percentage differentially expressed genes, are summarized in Table 1. Many genes that were

differentially regulated between the two strains of mice (groups A and B) are located on chromosome 12, predominantly in *Bps1* (8 in group A and 9 in group B) (3). All the 17 genes that were differentially expressed in the mouse strains C3H and HcB-28 and that are located in *Bps1* are presented in Table 2, and may be considered as candidate susceptibility genes. Of these genes, the strongest differences in gene expression between the mouse strains were found for a cDNA sequence in group A (*BC022687*, up to 3.7-fold higher in C3H mice) and for Immunoglobulin heavy chain 1 in group B (*Igh-1*, up to 3.6-fold higher expression in HcB-28 mice). Eight genes within *Bps1* (6 in group A and 2 in group B) showed a strong difference (i.e. at least two-fold) in gene expression between the two mouse strains, and map to the immunoglobulin heavy chain complex (*Igh*).

Of the 2,353 genes that were differentially expressed after *B. pertussis* infection, 1,702 genes were identical to the genes we have described previously to be regulated upon *B. pertussis* infection (2). Thus by examining additional mice, we identified approximately 650 additional genes regulated by *B. pertussis*, most of which were weakly up- or down-regulated. Most up-regulated genes upon *B. pertussis* infection (group D) are involved in immune- and inflammation-related processes or in generic processes, while most down-regulated genes (group C) are involved in non-immune processes such as muscle contraction.

Table 1: Gene-ontology based classification of genes with different expression

Category	Group A		Group B		Genotype (A+B)		Group C		Group D		Infection (C+D)		Total on array
Acute-phase response	0	(0%)	0	(0%)	0	(0%)	0	(0%)	13	(59%)	13	(59%)	22
Antigen presentation	0	(0%)	0	(0%)	0	(0%)	0	(0%)	23	(72%)	23	(72%)	32
Apoptosis	0	(0%)	3	(1%)	3	(1%)	9	(3%)	50	(15%)	59	(18%)	324
Cell cycle	0	(0%)	1	(0%)	1	(0%)	30	(6%)	59	(12%)	89	(17%)	510
Chemokine Activity	0	(0%)	0	(0%)	0	(0%)	1	(3%)	24	(67%)	25	(69%)	36
Mus musculus 12[a]	12	(2%)	11	(2%)	23	(4%)	34	(6%)	38	(7%)	72	(13%)	560
Bps-1 (Mus musculus 12)	8	(4%)	9	(5%)	17	(9%)	6	(3%)	8	(4%)	14	(8%)	185
Complement activation	0	(0%)	1	(3%)	1	(3%)	1	(3%)	8	(26%)	9	(29%)	31
Cytokine Activity	0	(0%)	0	(0%)	0	(0%)	8	(4%)	56	(29%)	64	(33%)	192
Cytoskeleton	0	(0%)	2	(0%)	2	(0%)	48	(9%)	45	(8%)	93	(18%)	531
Development	5	(0%)	9	(1%)	14	(1%)	137	(10%)	118	(8%)	255	(18%)	1433
Immune response	1	(0%)	2	(0%)	3	(1%)	6	(1%)	176	(43%)	182	(45%)	408
Inflammatory response	0	(0%)	1	(1%)	1	(1%)	5	(4%)	52	(43%)	57	(47%)	121
Metabolism	3	(0%)	20	(0%)	23	(0%)	300	(6%)	405	(8%)	705	(15%)	4771
Muscle contraction	0	(0%)	0	(0%)	0	(0%)	14	(29%)	2	(4%)	16	(33%)	49
Listed in any of the above	19		29		48		453		656		1109		6574
Other	3		22		25		383		513		896		5639
Unannotated	85		48		133		127		221		348		9744
Total	107		99		206		963		1390		2353		21957

[a]*Genes located on chromosome 12*

Hematologic and immunologic analyses in non-infected C3H and HcB-28 mice

To investigate possible differences in the basal immunological parameters between the two strains, we analyzed hematologic parameters, mitogen-induced splenocyte proliferation, and lymphocyte subset distributions. Because the two mouse strains displayed clear differences in basal gene expression, we wanted to examine if these differences led to alterations in blood cell composition or basal immune status. We observed a slightly higher number of platelets (P=0.0003) in the C3H/DISnA mice ($1.1*10^{12}$/l) compared to the HcB-28/Dem mice ($9.6*10^{11}$/l), and a slightly higher number of white blood cells (P=0.006) in the C3H/DISnA mice ($7.3*10^{9}$/l) compared to the HcB-28/Dem mice ($6.5*10^{9}$/l), but no differences in the numbers of red blood cells, reticulocytes, lymphocytes, neutrophils, eosinophils, and basophils (data not shown).

Splenocyte proliferation was determined by mitogen-induced ^{3}H-thymidine uptake. There was no difference in proliferation between the two strains after *ex vivo* stimulation with Con A (T-cell stimulus), LPS (B-cell stimulus) or Lectin (B- and T-cell stimulus) (data not shown). We observed no differences in the number of $CD19^{+}$ (B), $CD3\varepsilon^{+}$ (T), $CD4^{+}$ (Th) and $CD8^{+}$ (CTL) cells (data not shown).

Table 2: Candidate susceptibility genes.

Symbol	Max. fold[a]	*P*-value	Group[b]	Description
2610204M08Rik	1.5	0.0000000		RIKEN cDNA 2610204M08 gene
BC022687	3.7	0.0005453		cDNA sequence BC022687
LOC544906[c]	2.6	0.0024627		similar to monoclonal antibody heavy chain
LOC382694[c]	2.8	0.0043821		similar to immunoglobulin heavy chain
LOC211331[c]	2.7	0.0002482	A	similar to Ig H-chain
LOC238440[c]	2.7	0.0016035		similar to IgE antibody heavy chain (VDJ)
LOC238448[c]	3.2	0.0000868		similar to Igh-VJ558 protein
LOC544911[c]	2.1	0.0000310		similar to Ig heavy chain V region VH558 A1/A4 precursor
LOC432692	1.4	0.0005147		LOC432692
LOC544805[c]	3.5	0.0000050		similar to Ig heavy chain variable region precursor
1700001K19Rik	1.6	0.0002085		RIKEN cDNA 1700001K19 gene
Amn	1.7	0.0002930		amnionless
Ppp1r13b	2.2	0.0000019	B	protein phosphatase 1, regulatory (inhibitor) subunit 13B
Adssl1	1.5	0.0001518		adenylosuccinate synthetase like 1
Akt1	1.8	0.0000206		thymoma viral proto-oncogene 1
AI450948	1.5	0.0048316		expressed sequence AI450948
Igh-1a[c, d]	3.6	0.0000000		immunoglobulin heavy chain 1a (serum IgG2a)

[a]Maximum fold-change between genes that are located in Bps1 and that are significantly differentially expressed between C3H and HcB-28 mice.
[b]Hierarchical clustering as presented in figure 02, higher in C3H (A) or higher in HcB-28 (B).
[c]In the most recent annotation of the murine genome, eight probes map to the Immunoglobulin heavy chain complex (Igh) similar to Ig heavy chain variable region precursor.
[d]The oligo was designed based on accession number XM_484178 annotated as Igh-1a. Because this annotation is based on the reference C57BL/6 mice, the oligo can be considered as Igh-1b annotated.

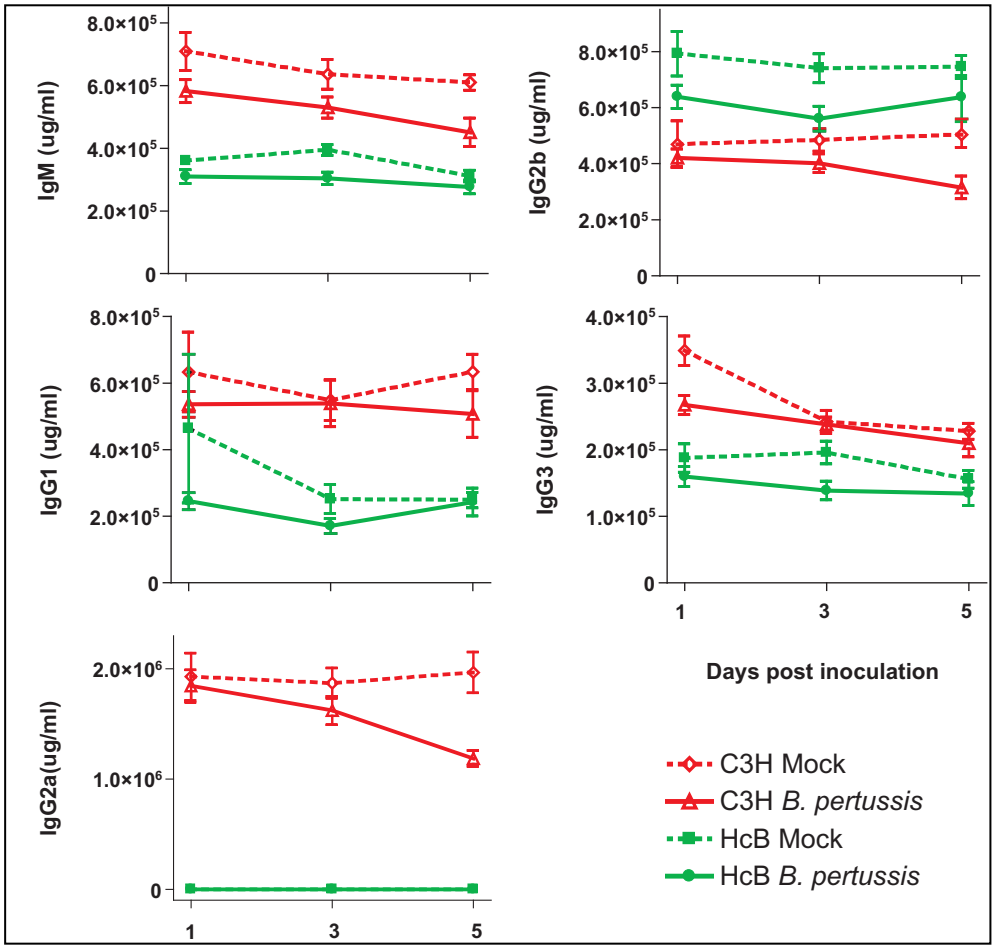

Figure 3: Serum levels of immunoglobulin classes after B. pertussis infection.
Twenty-four mice per group were inoculated with either B. pertussis or with culture medium only (Mock), mice were subsequently euthanized at one, three or five days post inoculation. Blood was collected for the measurement of serum levels of immunoglobulin classes. C3H mice are presented in red and HcB mice are presented in green. Dashed lines represent mock-inoculated mice and continuous lines represent B. pertussis-inoculated mice. Error bars represent the standard deviation. No significant difference (P>0.05, Bonferoni, ANOVA) was found between mock- or B. pertussis-inoculated mice of the same mouse strain, except for IgM at day 5 in C3H mice, IgG2a at day 5 in C3H mice, IgG2b at day 3 in HcB mice, IgG3 at day 1 in C3H mice and IgG3 at day 3 in HcB mice.All serum levels were significantly different (P<0.05, Bonferoni, ANOVA) between the two strains of mice except for IgM at day 5, IgG1 at day 1 and IgG2b at all days for B. pertussis infected HcB mice compared to C3H mice .

Finally we determined levels of IgM and subclasses of IgG in the sera of the mock- and *B. pertussis*-inoculated mice (Figure 3). Infection did not affect Ig levels compared to mock-infected mice of the same strain. We did, however, observe a significant difference in the Ig subset distribution between the two mouse strains (either mock- or *B. pertussis*-inoculated). C3H mice had significantly higher serum levels of IgM, IgG1, IgG2a and IgG3, while HcB-28 mice had significantly higher IgG2b levels compared to C3H mice. Importantly, HcB-28 mice had no detectable levels of IgG2a in the serum at all, which is consistent with the difference in gene expression at the *Igh-1* locus between the two mouse strains.

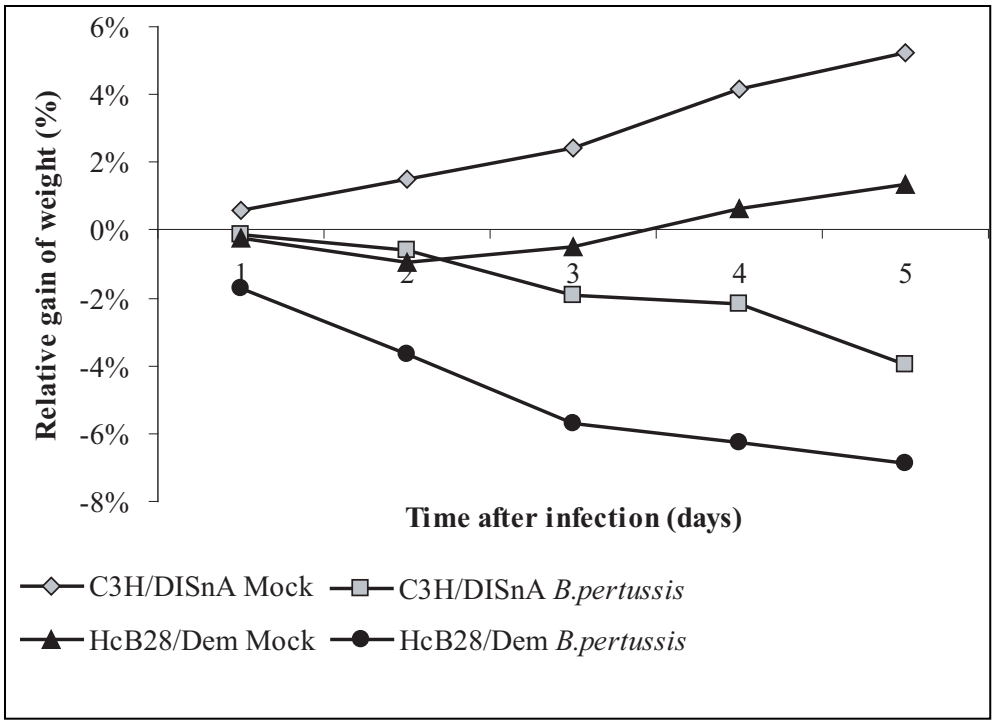

Figure 4: Relative gain or loss of weight after inoculation.
Twenty-four mice per group were inoculated with either B. pertussis or culture medium only (Mock) and body weights were measured daily.

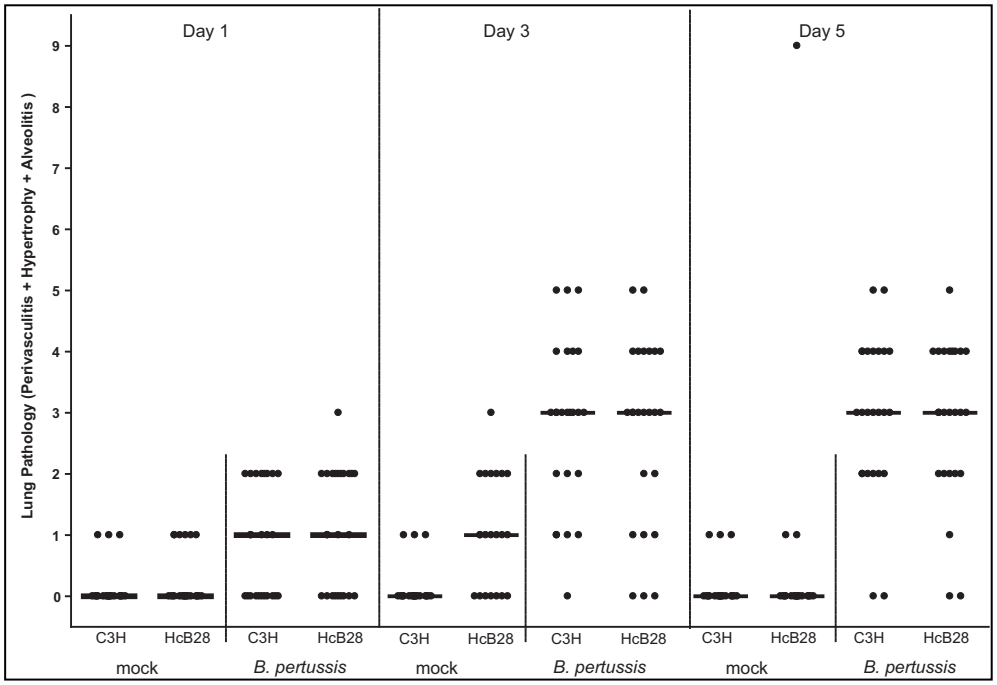

Figure 5: Summary of lung pathology post mock- or B. pertussis-inoculation.
H&E stained slides were examined for alveolitis, perivasculitis and hypertrophy of mucus-producing glands. Lung lesions were scored semi-quantitatively as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) per type of lesion and added up to calculate the pathology-score (range 0-15). Dots represent the pathology score per individual mouse, horizontal lines represent the groups median.

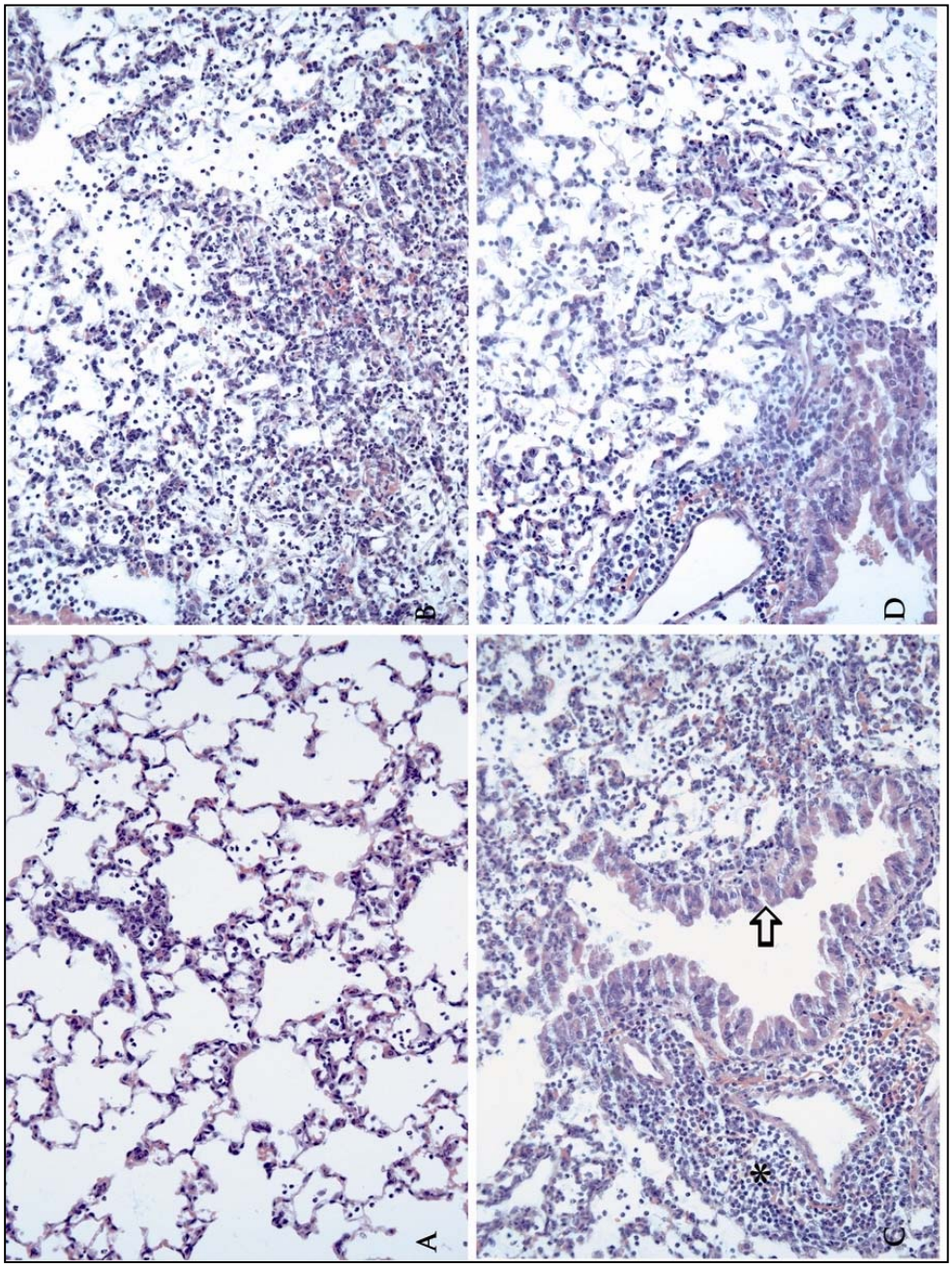

Figure 6: Examples of lung pathology post B. pertussis inoculation.
Lung sections (H&E, obj x 20) from B. pertussis infected mice. A: HcB28 day 1, mild alveolitis (grade 2 on scale from 1-5), as seen by inflammatory cells in alveolar septa and spaces. B: C3H day 3, alveolitis and thickened septa (pneumonia) varying from grade 3 (upper, right) to 5 (bottom). C: C3H day 3, asterix: perivasculitis grade 5, and arrow: hypertrophy bronchiolar epithelium, grade 3. D: HcB28 day 3, no major differences in pathology compared to C3H (B and C).

Clinical and pathological findings in *B. pertussis*-infected C3H and HcB-28 mice

To examine whether the two mouse strains differed in weight loss after infection, we determined their body weights after infection. Infected mice lost three to four percent of their body weight during the first five days post-inoculation, while all mock-inoculated mice gained weight during these five days (Figure 4). No significant differences in the gain or loss of weight were observed between the two strains, irrespective whether they were infected or not.

As a quantitative endpoint for inflammation, we determined lung weights relative to body weight. All mock-inoculated mice had relative lung weights of approximately one percent five days after inoculation, while *B. pertussis*-infected mice had relative lung weights of up to three percent. This difference in relative lung weights due to *B. pertussis* infection is significant from day one till day five post-inoculation, but was similar in both mouse strains (data not shown).

Histopathological evaluation of lung sections was performed as a second parameter of lung inflammation. Intranasal inoculation of *B. pertussis* causes an acute inflammatory response that is characterized by influx of polymorphonuclear leukocytes (PMNs) and macrophages, starting in the perivascular and peribronchiolar areas on day one, and extending to alveolar walls and lumina on days three and five. No differences were observed in the histopathological lesions findings between the two mouse strains (Figure 5 and 6).

Discussion

Studying genetic differences in susceptibility to *B. pertussis* infection may point to novel insights in the pathogenesis of this infection. We have previously identified *B. pertussis susceptibility locus-1* (*Bps1*) in HcB-28 mice (3). The *Bps1* locus is located on chromosome 12, spanning a region of 185 genes and has a dominant positive effect on the clearance of *B. pertussis* from the lung. In this study we examined gene expression profiles in HcB-28 and C3H mice, which differ in their susceptibility to *B. pertussis.* Twelve and a half percent of the genomes of these mice are from a different genetic background (12,31). The traditional approach for identification of relevant genes in susceptibility loci is a combination of positional cloning and linkage analysis (26,28). This method has proven to be effective (8,27), but has disadvantages. We have therefore chosen for an alternative strategy. Using this approach we attempted to identify candidate susceptibility genes that control the difference between these two mouse strains. We hypothesized that the difference in susceptibility to *B. pertussis* infection could (partly) be explained by a different gene expression profile between the mouse strains.

We have previously shown that *B. pertussis* infection in C3H mice induces a wide transcriptional response, which appears to be partly specific for *B. pertussis* and partly non-specific (2). This study revealed that 1,841 genes are differentially expressed in the lungs of mice after *B. pertussis* inoculation, and most up-regulated genes are involved in immune- and inflammation-related processes or in generic processes, while most down-regulated genes are involved in non-immune processes. In the present study, we found that HcB-28 and C3H mice showed a similar gene expression profile upon infection and identified approximately 650 additional genes regulated by *B. pertussis*, most of which were weakly up- or down-regulated. However, the gene expression profiles and enrichment for GO categories were identical as described in the previous study. A substantial number of genes and pathways suggest a central role of PMN recruitment and activation in the pathogenesis of *B. pertussis* infection. The transcriptional profiles further indicate in particular the significance of TLR activation and apoptosis (2). The reason for the detection of the 650 additional genes, besides the strain differences, is that by adding an extra mouse strain the number of samples doubled increasing the power of detection. Hundred and thirty-nine genes which we have described previously to be regulated upon *B. pertussis* infection were not detected in the present study. These genes were borderline significantly regulated (median FDR of 0.03) and only slightly induced (median 1.4-fold). These genes are therefore probably less important in the host response to *B. pertussis* infection. The finding that this list of 139 genes does not show significant enrichment for any GO-term, including immunological terms, corroborates this.

Although the two mouse strains differ in 12.5% of their genome (12.5% of the genomes of these mice are from a different genetic background), we observed

no marked differences in their phenotypical characteristics other than the previously observed difference in bacterial numbers in the lungs after infection (Figure 1b). C3H mice did have slightly higher numbers of circulating platelets and white blood cells compared to the HcB mice, but the cellular proportions, as well as the proliferation of splenocytes was identical for both strains. There was also no significant difference observed in body weight, lung weight and histopathological findings in response to *B. pertussis* infection between the two mouse strains. The major difference is that HcB-28 mice did not have detectable IgG2a serum levels.

We observed 206 genes that were differentially expressed between the two mouse strains, but these genes were identically expressed in mock- or *B. pertussis*-inoculated mice. The majority of these genes (65%) are unannotated. These unannotated genes tend to have no GO functional annotation, because they are not "regular" protein-coding genes and many of them are not (sufficiently) mapped to a chromosomal locus to warrant including them under chromosome 12 or *Bps-1*. The mentioned 23 genes, which were mapped to chromosome 12, are significant at P = 5.29e-014 (Fisher exact probability, Bonferroni correction for multiple testing). The same test applied to all other mouse chromosomes yielded P values > 0.05. Twenty-three out of the 206 genes were located on chromosome 12, which can be explained by the fact that approximately one fifth of the genetic variation between the two mouse strains is due to variation on chromosome 12 (3). Because the genes that were differentially regulated between the two mouse strains only showed differences in expression before infection, it appears likely that such intrinsic differences in gene regulation are involved in determining differences in susceptibility to *B. pertussis* infection. Alternatively, such genetic differences may be explained by genes that are not differentially regulated between these two strains of mice, or by processes at present not fully characterized and possibly involving differential expression of genes by mechanisms such as microRNA's. Remarkably, 17 of these genes were located in the *Bps1* region, 8 of which mapped to the *Igh* complex. Among these 8 genes were the *Igh-1* gene and genes that encode for Ig heavy chain variable regions. We observed a significantly higher expression (up to 2.8-fold) of the gene variant of *Igh-1* isotype b in HcB mice compared to C3H mice. The Igh locus is genetically polymorphic and very complex (30). The *Igh-1* gene exists in 2 major genetic variants (*Igh-1a* and *Igh-1b*) with 83.8% similarity (20). The *Igh-1a* allele codes for the heavy chain of IgG2a while the *Igh-1b* allele codes for the heavy chain of IgG2c (19). Mouse strains such as C57BL/6 and C57BL/10 (the donor strain of the HcB mice) only contain the gene variant *Igh-1b* and are therefore incapable of producing IgG2a, while mouse strains such as BALB/c only contain the gene variant of *Igh-1a* and are therefore incapable of producing IgG2c (19,20). The oligo for *Igh-1* spotted on the microarray, was designed based on accession number XM_484178 annotated as *Igh-1a.* Because this annotation is based on the reference C57BL/6 mice, this

oligo can be considered as *Igh-1b* annotated. In the sera of HcB mice we detected no IgG2a while C3H mice had significant titers of IgG2a, thereby confirming the expression results. It is tempting to speculate if and how genes within the *Igh* complex may affect differences early in the course of *B. pertussis* infection. Possible mechanisms may include differences in transcriptional gene regulation affecting immune responsiveness, different function of the IgG2a or c isotypes, or different usage of V chains. This latter possibility might imply the existence of "natural antibodies" reacting with *B. pertussis* epitopes. It has previously been shown that genes within the *Igh-1* locus are predominantly associated with the course of a herpes simplex virus type-1 (HSV-1) infection in mice by an unknown mechanism (11,23,32). Pro-inflammatory cytokines such as IL-1β, IL-4, IL-6 and IL-7 participate in this infection (1). Interestingly, natural killer cell activity appears to be regulated by the *Igh-1* locus but could not simply explain the differences in HSV-1 susceptibility (32). *Igh*-linked genes have further been implicated in T suppressor cell activity (15,22).

Conclusions

In this study we reduced the number of candidate susceptibility genes within the *Bps1* locus by microarray analysis. Gene expression changes upon *B. pertussis* infection appear highly identical between C3H and HcB-28 mouse strains despite the different course of *B. pertussis* infection in these strains. Because the genes that were differentially regulated between the mouse strains only showed differences in expression before infection, it appears likely that such intrinsic differences in gene regulation are involved in determining differences in susceptibility to *B. pertussis* infection. Alternatively, such genetic differences in susceptibility may be explained by genes that are not differentially regulated between these two mouse strains or by processes other than differential gene expression. Genes in the *Igh* complex, among which *Igh-1*, may be likely candidates to explain differences in susceptibility to *B. pertussis.* Further work should establish the role of the *Igh* complex in *B. pertussis* infection and determine its mode of action.

Supplementary data

Raw data as well as the detailed description of the experiment was uploaded to the freely accessible online database ArrayExpress http://www.ebi.ac.uk/arrayexpress/. Expression levels of all 2,559 genes are presented in the supplementary data.

Authors contribution

SB: carried out the infection and microarray studies and wrote the manuscript. RJV: participated in the study design and coordination and helped to draft the manuscript. JLAP: participated in the design of the microarray analysis and performed the statistical analysis. ERG: carried out the immunoassays. PWW:

evaluated the lung pathology. TMB: participated in the design of the microarray analysis. PD: responsible for the genetic model of recombinant congenic mice. HJK, FRM, and BH: participated in the study design and coordination. TGK: conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Chapter 5

Host genetics of *Bordetella pertussis* infection in mice; the significance of Tlr4 in genetic susceptibility and pathobiology.

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Abstract
Susceptibility to and severity of *Bordetella pertussis* infections in infants and children varies widely, suggesting that genetic differences between individuals influence the course of infection. We have previously identified three novel loci that influence the severity of whooping cough using recombinant congenic strains of mice, *Bordetella pertussis susceptibility locus 1, 2 and 3 (Bps1, 2 and 3)*. Because these loci could not account for all genetic differences between mice, we extended our research for additional susceptibility loci. We therefore screened eleven inbred strains of mice for susceptibility towards a pertussis infection after intranasal infection. Susceptibility was defined by the number of bacteria in the lungs, being indicative for the effect between the clearance and replication of bacteria. The most resistant (A/J) and the most susceptible (C3H/HeJ) strains were selected for further genetic and phenotypic characterization.
Linkage between bacterial clearance and chromosomal location was investigated using 300 F2 mice, generated by crossing A/J and C3H/HeJ mice. We found linkage between delayed clearance of bacteria from the lung and a large part of chromosome 4 in F2 mice with a maximum LOD-score of 33.6 at 65.4 Mb, which is the location of *Tlr4*. C3H/HeJ mice carry a functional mutation in the intracellular domain of *Tlr4*. This locus accounted for all detectable genetic differences between these strains.
Compared to A/J mice, C3H/HeJ mice showed a delayed clearance of bacteria from the lung, a higher relative lung weight, and increased body weight loss. Splenocytes from infected C3H/HeJ mice produced almost no IL1-β and TNF-α upon *ex vivo* restimulation with *B. pertussis*, compared to A/J mice, and also showed a delayed IFN-γ production. TNF-α expression in the lungs three days after infection was 5-fold increased compared to non-infected controls in A/J mice and unaffected in C3H/HeJ mice.
In conclusion, *Tlr4* is a major host factor explaining the differences in the course of infection between these inbred strains of mice. Functional *Tlr4* is essential for an efficiënt IL1-β, TNF-α and IFN-γ response, efficient clearance of bacteria from the lung, and reduced lung pathology.

Introduction

The clinical course of *Bordetella pertussis* infection varies widely. Knowledge about host genetic and immunological factors that influence susceptibility and severity of infection, may lead to the identification of new approaches for prevention or treatment of this disease (13). Knowledge of human genetic factors that influence *B. pertussis* infection is still very limited. A number of studies have provided clues for the role of host genes in susceptibility of mice to *B. pertussis* infection (4,11,19,23). We have recently shown that host genes of mice affects the clearance of bacteria from the lung and that *B. pertussis* infection is under multigenic control. This study resulted in the identification of three novel loci (*Bps1 – 3)* that influence the clearance of *B. pertussis* from the lung These loci could explain up to 10% of the variation in the lung colonization observed in F2 mice Therefore additional loci are likely to influence the course of *B. pertussis* infection (2). In the present study, we used inbred strains of mice to identify additional genetic factors.

In addition to *Bps1-3,* others identified the Toll Like Receptor 4 (*Tlr4*) as a major factor that influences the course of *Bordetella bronchiseptica* and *B. pertussis* infection in mice (11,20,21). In these studies C3H/HeJ mice were used. These mice carry a functional mutation in the gene coding for Tlr4 rendering them unresponsive to lipopolysaccharide (LPS) (6,26).

The aims of the present study were, first, to examine whether inbred strains of mice show genetic differences in susceptibility to *B. pertussis* and second, whether we could identify genetic loci responsible for such differences, and third, to examine the pathobiological mechanisms of the identified susceptibility loci. To this aim, we compared the present approach using inbred strains of mice to our previous approach using recombinant congenic strains of mice. We examined lung colonization, lung pathology, and the immune responses of inbred strains after infection to correlate phenotypic characteristics of infection with the genetic background of the mice.

Materials and Methods

Experimental design

We examined the course of *B. pertussis* infection in 11 inbred strains of mice. Approximately 8 animals of each strain were initially used to determine the number of bacteria in the lung one week post infection. A total of 300 F2 hybrid mice were obtained by crossing the strain with the highest number of bacteria in the lung (C3H/HeJ) with the strain with the lowest number of bacteria in the lung (A/J) and subsequently intercrossing their F1 progeny. The 300 F2 hybrid mice were examined as described below. Due to logistical limitations we infected 50 mice per day and combined the results. To assure the reproducibility of the infection model, BALB/c mice were included on each day of infection and infected in the same way. The original inbred strains of mice were examined on three different days. The F2 hybrid mice were examined on six different days.
To confirm that differences in lung pathology and clinical response were caused by Tlr4, *Tlr4* defective ($Tlr4^{Lps\text{-}d}$) C3H/HeJ mice, *Tlr4* wild-type ($Tlr4^{Lps\text{-}n}$) A/J and C3H/HeOuJ mice were infected with *B. pertussis* as described below. To control groups (mock) a similar volume of Verwey medium was given.
The number of bacteria in the lungs of the BALB/c control mice, one week post-infection, was similar regardless of the day the experiment was performed. Because there was no significant difference between any of the control groups ($p>0.05$), we combined the results of all experiments (data not shown).

Animals

Female mice were used for the infection experiments. A/J, C57BL/6J, SPRET/Ei, Cast/Ei, DBA/2J, B10.D2.H2/oSnJ, AKR/J, BALB/cJ, 129X1/SvJ, C3H/HeOuJ and C3H/HeJ strains of mice were supplied by the Jackson Laboratory (JAX, Bar Harbor, USA). BALB/cOlaHsd (referred to as BALB/c) mice were supplied by Harlan (Harlan Europe, Horst, The Netherlands). F2 hybrid mice were generated by crossing A/J mice with C3H/HeJ mice, and subsequently intercrossing their F1 progeny. Mice were acclimatized for at least one week before the start of the experiments. Mice received standard laboratory chow (SRM-A, Hope Farms, Woerden, the Netherlands) and tap water ad libitum. All animal experiments were approved by the Institute's Animal Ethics Committee.

Bacteria

B. pertussis strain B213, a Tohama derivative (ptxA1,prn1), and *B. pertussis* strain B2566, a clinical isolate from 1997 (ptxA2, prn2) (14,25) were cultured by plating on Bordet-Gengou agar supplemented with 15% sheep blood and 30μg/ml streptomycin. Plates were incubated for 4 days at 35°C. The number of colony forming units (CFU) was determined using a ProtoCOL SR Colony counter (Synbiosis, Camebridge, UK). All dilutions of bacteria were made in Verwey medium (NVI, Bilthoven, the Netherlands).

Infection experiments

The number of viable *B. pertussis* bacteria was determined in the lung one week after infection (14,35). Briefly, mice were intranasally infected with $2*10^7$ colony forming units (CFU) of *B. pertussis* after being anaesthetized with isoflurane. Seven days after infection, mice were sacrificed and the lungs were collected. A ligature was made around the right bronchus, after which the right lobes were removed for counting of bacteria (34). The remaining left lung lobe were fixed intratracheally using 4% formalin for histological examination. The right lung lobes were homogenized in Verwey medium, and diluted 10 and 1,000 times. The number of CFU in these dilutions was determined by plating on Bordet Gengou agar supplemented with 15% sheep blood and 30μg/ml streptomycin. Plates were incubated for 4 days at 35°C before counting the number of bacteria using a ProtoCOL Colony counter (Synbiosis).

Macroscopic and histopathologic examination

Mice were weighed one week before infection, and three, seven, fourteen and twenty-one days after infection to determine the relative gain or loss of weight. Lung weights were determined after sacrifice of mice as a parameter for lung inflammation. Lung weights are represented relative to the body weights.

Formalin-fixed lungs were embedded in paraplast (Monoject). Transverse sections of 5 μm were stained with hematoxylin-eosin. In a blinded fashion, an independent observer examined the slides for peribronchiolitis (i.e., infiltration of inflammatory cells in the peribronchiolar space), alveolitis (i.e., infiltration of inflammatory cells in the alveoli), perivasculitis (i.e., infiltration of inflammatory cells in the perivascular space), hypertrophy of mucus-producing glands, free protein and eosinophilia. Lung lesions were scored semi-quantitatively as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5), as previously described (3).

Genotyping

Genomic DNA was isolated from mice tails using the DNeasy Tissue kit (Qiagen). The sequences of all primers were obtained from the mouse genome database of the Massachusetts Institute of Technology (MIT) (24). DNA was amplified in 10 μl volume using 5 μl hotstar 2x Mastermix (Qiagen), 1.0 μM of each primer and approximately 10ng tail-DNA. 6-Carboxyfluorescein (FAM)-labeled microsatelite primer-sets were used (Isogen Life science, Maarssen, the Netherlands). Amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems), as follows: 15 min at 95°C to denature the DNA and activate the Hotstar Taq, 30 cycles of 45 sec at 94°, 45 sec at 57°C and 1 min at 70°C, and finally 10 min at 72°C. Fragment sizes were determined on a 3700 Capillary DNA Sequencer/Genotyper system (Applied Biosystems) using Genotyper software (Applied Biosystems). The single nucleotide polymorphism (SNP) markers were analyzed by Restriction Fragment Length Polymorphism (RFLP)-

assay on a 2.5% agarose gel (Table 1). PCR was performed as described above, restriction conditions were used according to the manufacturer's instructions (New England Biolabs).

Cytokine responses

To examine cytokine production by spleen cells after infection, single-cell suspensions were prepared from the spleen by pressing the tissue through a cell strainer (Falcon). The cells were washed once in RPMI 1640 (Gibco BRL, Life Technologies). The splenocytes ($1.5*10^5$ cells per well) were cultured (37°C, 5% CO_2) in 96-wells tissue culture plates (Nunc, Denmark) in the presence of concanavalin A (ConA) (5 µg/ml, Sigma Chemical Co.) or *B. pertussis* ($1.0*10^5$ heat-inactivated bacteria per well). Bacteria were heat-inactivated at 56°C during 30 min. The culture medium was composed of RPMI 1640 (Gibco BRL, Life Technologies), supplemented with 2 mM glutamine, penicillin, streptomycin, HEPES buffer, β-mercaptoethanol (50 mM) and 10% FCS. After 72 h, culture supernatant was collected and stored at −80°C until analysed for cytokine production (5,33).

IL1-β, IFN-γ and TNF-α concentrations were determined by ELISA (Biosource International) using the concentrations recommended by the manufacterer. Briefly, 96-well plates (Nunc-Immuno Plate) were coated with 1.25 µg/ml anti-mouse IL1-β, IFN-γ or TNF-α in coating buffer (0.05 M carbonate buffer, pH 9.6, Sigma). After overnight incubation at 4°C, the plates were incubated in blocking buffer (1% BSA in Tris buffered saline, Sigma) for 2 h at room temperature (RT), and washed (0.05% Tween-20, Merck, the Netherlands). Recombinant mouse cytokines (Biosource) were used as a standard. Standards, as well as serial dilutions of splenocyte culture supernatants, were added to the plate. Plates were incubated at 37 °C for 2 h and washed. Biotinylated anti-mouse IL1-β, IFN-γ or TNF-α (0.125 µg/ml) was added, and incubated for 1 h at RT. The plates were washed, and horseradish peroxidase-labeled streptavidin (10000-fold dilution, Strepta-E+, Central Laboratory of the Blood transfusion service, Amsterdam, the Netherlands) was added and incubated for 1 h at RT. After washing, Tetramethylbenzidine solution (0.1 mg/ml TMB, Sigma-Aldrich) plus 0.006% H_2O_2, in 0.1 M Sodium Acetate (pH 5.5) was added. The color reaction was stopped by adding Sulfuric acid (10%, Merck). The plates were measured at 450 nm on a fluostar platereader (Fluostar galaxy, BMG Labtech, Germany)(32).

Table 1: SNP assays

	Chromosome	Position (Mb)	Forward primer	Reverse Primer	SNP Allele		Restriction Enzyme
					C3H	A/J	
Rs4224427	4	32	caaagaggctgaagcacttg	gtctaggaccttcccacaa[a]	A	G	SspI
rs3023006*(Tlr4)*	4	65,4	gctttcacctctgccttcac	ataaccttccggctcttgtg	A	C	Hsp92 II
Rs3022979	4	75,5	ataatggggctaacgcaatg	gaagagggcatcagtgttcc	A	C	BsrI

[a] *Mismatch primer was used to create a restriction site for SspI.*

TLR4 and TNF-α mRNA expression

To examine mRNA expression, lungs from infected animals were incubated overnight in RNA-later (Qiagen) at 4°C, and stored at -80°C. Lungs were homogenized in RLT-buffer (Qiagen) with a rotor homogenizator, and RNA was extracted with a RNeasy kit (Qiagen) as described by the manufacturer. cDNA was generated using the High Capacity cDNA archive kit containing random hexamer primers (Applied Biosystems). mRNA expression was measured using Taqman gene expression assays (Applied Biosystems) on a 7500 Fast Real-Time PCR System. We used assays on demand for *Tlr4* (Mm00445274_m1) and TNF-α (Mm00443258_m1). For the reference gene, RNA Polymerase IIa (Polr2a), the assay was designed using the primer express program (Applied Biosystems) resulting in probe CATCCGCTTCAATTCAT, forward PCR primer GCAGTTCGGAGTCCTGAGT, and reverse PCR primer CCCTCTGTTGTTTCTGGGTATTTGA. Taqman probes carried a 5' FAM reporter label and a 3' Non Fluoro quencher(NFQ) group. Taq polymerase was activated by heating for 20 sec at 95°C. Amplification was for 3 sec at 95°C and 30 sec at 60°C for 40 cycles in a Taqman Fast Universal PCR Master mix (Applied Biosystems) containing 5 µl of cDNA template, 1µl TaqMan Gene Expression Assay mix (20X mix containing primers and probes) in a total volume of 20 µl. The fluorescence intensity of the reporter label was normalized to the rhodamine derivative ROX as a passive reference label present in the buffer solution (9,30). The relative concentration of the *Tlr4* and TNF-α mRNA was determined by the comparative threshold cycle method (ddCt) (1,9,30). Each sample was run in triplicate.

Statistical analysis

The differences in numbers of CFU in the lungs between the different inbred strains of mice were examined by Analysis of Variance (ANOVA, SPSS) and tested with the Student-Newman-Keuls test for multiple comparisons or Bonferoni post hoc test. The Bonferoni post hoc test is known to be the most conservative test for analysis of variance (ANOVA) analysis. The test is similar to for instance the Fisher's least significant difference test (LSD), but the observed significance levels are adjusted for the number of comparisons made. If a difference is significant according to the Bonferoni, it is for all other tests. The Student-Newman-Keuls test was used to test the difference between multiple groups. To stabilize variances and to obtain approximately normal distributions, the CFU's were square root (sqrt) or natural definition (ln) transformed. In F2 mice, linkage between the CFU's in the lung and the genetic markers, and the effect on the total phenotypic variation, were calculated by ANOVA with genotype as fixed factor, and CFU as dependent variable. To correct for the influence of experiment, experiment was included as random factor. All single markers and all pairs of non-linked markers were tested for linkage with another marker or interaction between markers. Interaction, or epistasis, is defined as the combined effect of two or more genes on a phenotype that could not have been predicted as the sum of their separate effects (8). Linkage is presented as p-value and Log of the odds (LOD) score. The latter was calculated as –log of the significance (p-value).

All markers and interactions were tested at the level of 0.05 ($p<0.05$). p-values were corrected for multiple comparisons using the formula (15,16) :

$$P_{corrected} = [C+2\rho GT^2]P$$

Where $P_{corrected}$ is the desired corrected p-value, C is the number of chromosomes segregating in the cross (20 for mice), ρ is the crossover rate (1.5 for a F2 hybrid generation), G is the genome length of the segregating part of the donor genome in Morgans (the mouse genome is 16M), T^2 is the threshold (the F-value from ANOVA for the observed p-value is used as T^2) and P is the observed uncorrected p-value.

The estimated effect of a linked locus on the total of the observed phenotypic variation is presented as R^2 (ANOVA, SPSS). To test the reproducibility of the infection protocol, we performed a T-test between the CFU's of the control mice infected in different experiments.

Difference in survival of animals was calculated by Kaplan Meier analysis (Survival, SPSS). We used time after infection when death occurred as Time, Death as status, and Group as factor.

Results

Colonization

To examine whether inbred strains of mice differ in susceptibility to *B. pertussis*, we examined 11 different inbred strains of mice. The number of viable *B. pertussis* bacteria in the lung one week after infection was used as phenotype. The number of CFU varied from $2.4*10^5$ to $1.2*10^7$ CFU per lung (Figure 1), therewith confirming the genetic basis for modulation of the course of *B. pertussis* infection. Significant differences in lung colonization were observed between some of these strains of mice. From these strains we selected A/J as most resistant strain and C3H/HeJ as most susceptible strain to generate an F2 intercross generation.

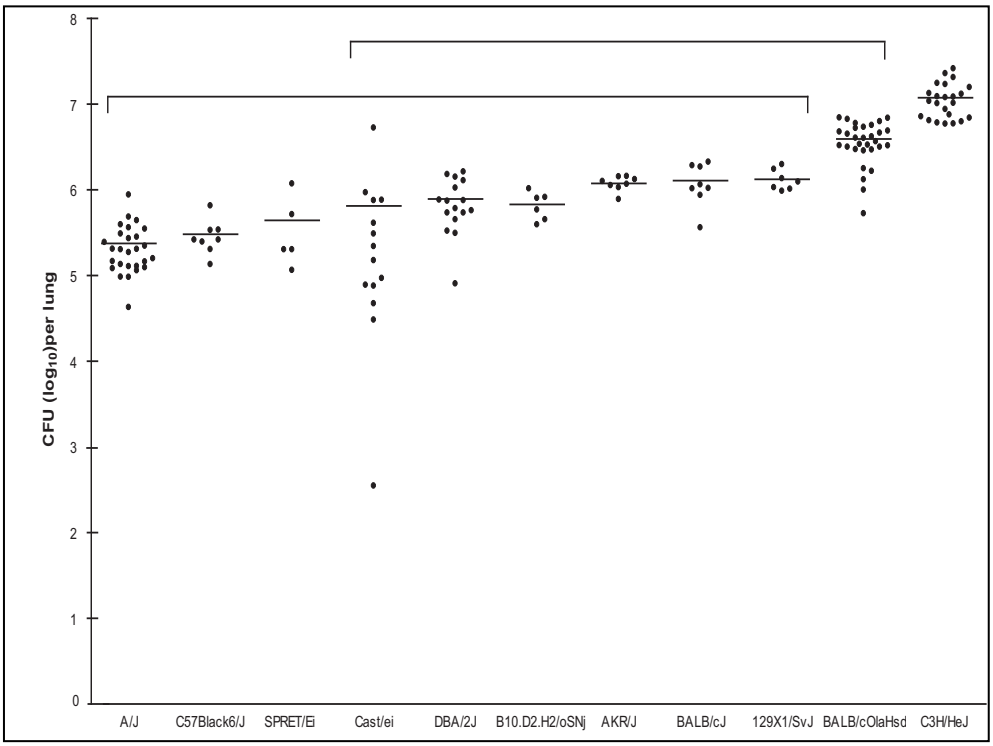

Figure 1: Colonization of inbred strains of mice by B.pertussis.
Inbred strains of mice were infected intranasally with B.pertussis strain B213, and seven days later lungs were removed, and the number of viable B.pertussis (B213) was determined. Each dot represents the number of bacteria in the lung of an individual mouse. Horizontal lines indicate the group average. Upper horizontal lines connect groups of mice who are mutually not significantly different according to the Student Neuman Keuls test. From these strains, A/J and C3H/HeJ were selected for generating F2 hybrid generations.

Linkage analysis

To search for genetic loci that could explain the differences between C3H/HeJ and A/J mice, 300 (A/J x C3H/HeJ) F2 hybrid mice were infected and examined as described above. As expected, the F2 mice showed a greater variation in the numbers of bacteria compared to the parental strains (Figure 2). The number of CFU per lung ranged from 1 x 10^2 (detection limit) to almost 1 x 10^8. The average number of CFU was 4,5 x 10^6. All mice were individually genotyped, and we compared the genotypes with phenotypes to identify possible susceptibility loci by linkage analysis.

We started testing markers in the *Bps1* region (2) and a mutation in the *Tlr4* gene that was described in C3H/HeJ mice (11,21). We found no linkage with markers from the *Bps1* region (data not shown), but strong linkage was found with the *Tlr4* locus with a LOD-score of 33.6 (P=2.324 x 10^{-34}, $P_{corrected}$=1.188 x 10^{-30}). Additional markers surrounding this gene were tested and almost all markers located on chromosome 4 showed linkage with reduced clearance of bacteria from the lungs, with a maximum LOD-score of 33.6 at 50.3cM, which is the location of *Tlr4* (Figure 3). Forty-five percent (r^2=0.451) of the variation in the number of bacteria in the experiments could be ascribed to *Tlr4*. Due to logistical limitations the F2 hybrid mice were examined in six experiments. To assure the reproducibility of the infection model each experimental group contained BALB/c mice that were infected in the same way. There was no significant difference in CFU's in BALB/c mice between different experiments (*P=0.365)*. However, the residual mean square error of the results in the BALB/c mice (ε^2 = 1.35 x 10^6) is larger than the residual mean square error of the *Tlr4* genotype (ε^2 = 1.07 x 10^6), which means that the residual variation is due to environmental variation. Thus, virtually all of the genetically detectable variation in lung clearance in these experiments could be ascribed to the *Tlr4* gene. Though we set out to test approximately 5 markers per chromosome, we decided that further testing was no longer informative.

Influence of bacterial genetic differences

To examine whether genetic differences in *B. pertussis* also affect the course of infection in these mice, we infected A/J and C3H/HeJ mice with *B. pertussis* strain B213 and a recent clinical isolate (B2566). The two strains differ with respect to pertussis toxin and pertactin. The number of bacteria in the lungs of mice infected with *B. pertussis* strain B2566 was similar to the number of bacteria in the lungs of mice infected with *B. pertussis* strain B213. A/J mice showed an average of 2*10^5 bacteria per lung while C3H/HeJ mice showed an average of 2*10^7 CFU per lung (Figure 4). So, the observed difference between these inbred strains of mice does not appear to be *B. pertussis* strain-specific.

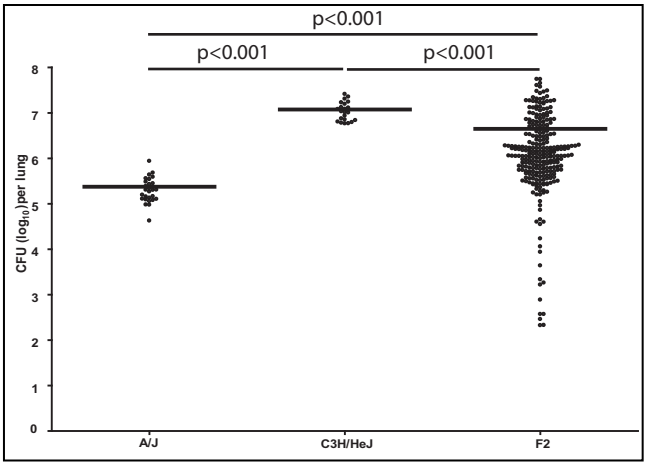

Figure 2: Colonization of B213 in A/J x C3H/HeJ F2 mice.
The F2 generation, obtained by crossing C3H/HeJ mice with A/J mice and subsequently intercrossing their F1 progeny, was infected intranasally with B.pertussis. Seven days after infection, lungs were removed, and the number of viable B.pertussis was determined. Each dot represents the number of bacteria in the lung per individual mouse. Horizontal lines indicate the group average. Horizontal connecting lines represent the level of significance of difference between groups. The experiment was performed twice and a representative result was shown.

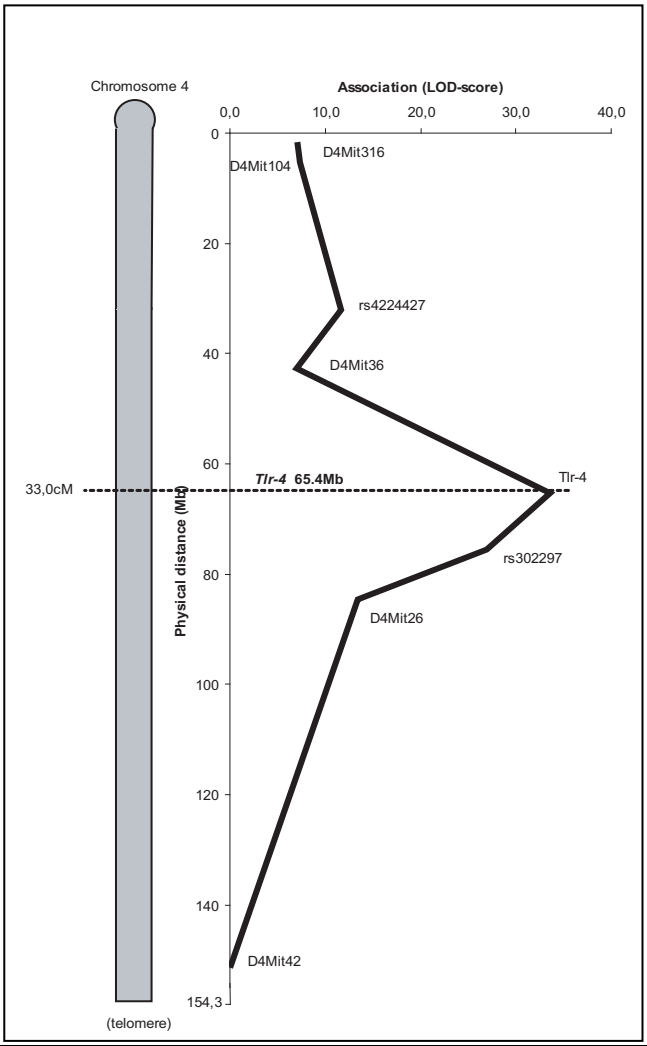

Figure 3: Linkage between the degree of colonization and markers on chromosome 4.
Linkage (LOD-score) between the degree of lung colonization by B.pertussis (phenotype) and chromosomal loci (genotype). The association was calculated by ANOVA with genotypes as fixed factor, and square root CFU as dependent variable. The LOD score is plotted as -log p against the physical distance of chromosome 4. The region, has a maximum LOD-score of 33.6 ($p=2.324*10^{-34}$, R^2 = 0,451) for the p712h mutant in Toll like receptor 4(Tlr4).

Time course of colonization

Because *Tlr4* appeared to be a dominant genetic factor, we subsequently characterized the role of Tlr4 in more detail. We first examined the number of bacteria at different time points after infection in different strains of mice.

The number of bacteria in the lung was determined 3, 7, 14 and 21 days after infection of A/J(*Tlr4*$^{Lps\text{-}n}$), C3H/HeOuJ(*Tlr4*$^{Lps\text{-}n}$) and C3H/HeJ (*Tlr4*$^{Lps\text{-}d}$) mice (Figure 5). C3H/HeOuJ mice are genetically identical to C3H/HeJ mice except for the mutation in the *Tlr4* gene (31). A/J and C3H/HeOuJ showed the same course of clearance of bacteria from the lung, while C3H/HeJ mice showed a significantly slower clearance as evidenced by a higher number of CFU's per lung up to 14 days post-infection. Twenty-one days after infection, almost all bacteria were cleared from the lung in all groups of mice.

Clinical observations.

To characterize differences in the clinical course of infection between C3H/HeJ (*Tlr4*$^{Lps\text{-}d}$) and A/J (*Tlr4*$^{Lps\text{-}n}$) mice, 24 mice of both strains were inoculated with *B. pertussis* ($6.1x10^7$CFU per mice). Unexpectedly several animals died after infection (Figure 6). Nine A/J mice died within the first four days after infection, while eleven C3H/HeJ mice died after four days.

All mice were weighed one week before infection, and 3, 7, 14 and 21 days after infection. Differences in body weight are presented in Figure 7. Infected mice lost 5 to 15 percent of their body weight in the first seven days after infection. Fourteen days after infection, A/J mice had regained most of their initial weight, while C3H/HeJ mice were still losing weight. Twenty-one days after infection all mice had regained their weight. Thus, in these two strains of mice, mortality appears to be inversely related to weight loss after infection, i.e. A/J mice died earlier, but surviving mice recovered quicker compared to C3H/HeJ mice.

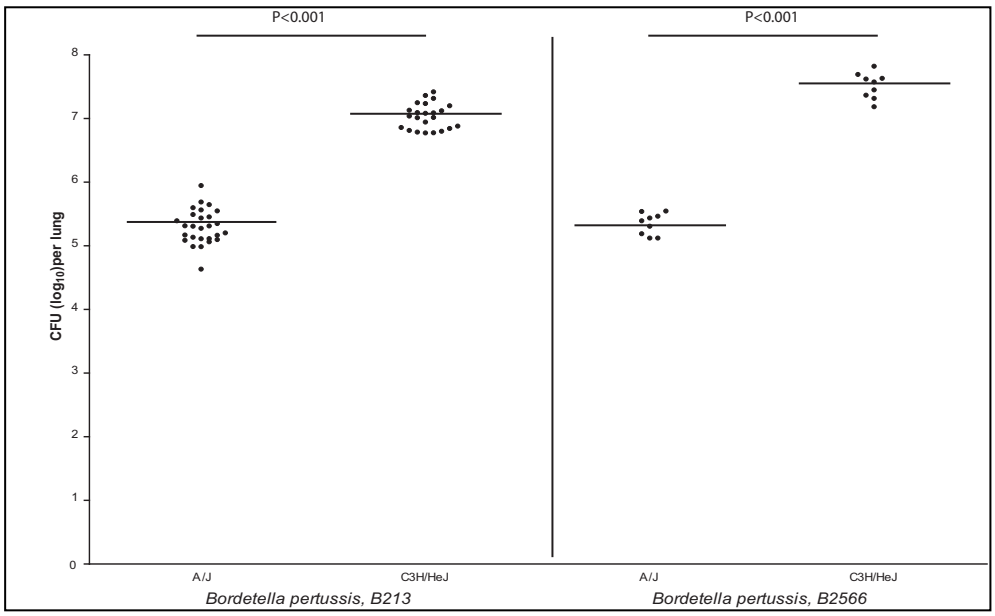

Figure 4: Colonization in two inbred strains of mice by distinct B.pertussis strains.
Seven days after inoculation with B.pertussis B213 (Tohama) and B2566 (a recent clinical isolate), lungs were removed, and the number of viable B.pertussis was determined. Each dot represents the number of bacteria in the lung of an individual mouse. Horizontal lines indicate the group average. Horizontal connecting lines represent the level of significance of difference between groups. Mice inoculated with B.pertussis B213 are displayed in the left panel, mice inoculated with B.pertussis B2566 are displayed in the right panel.

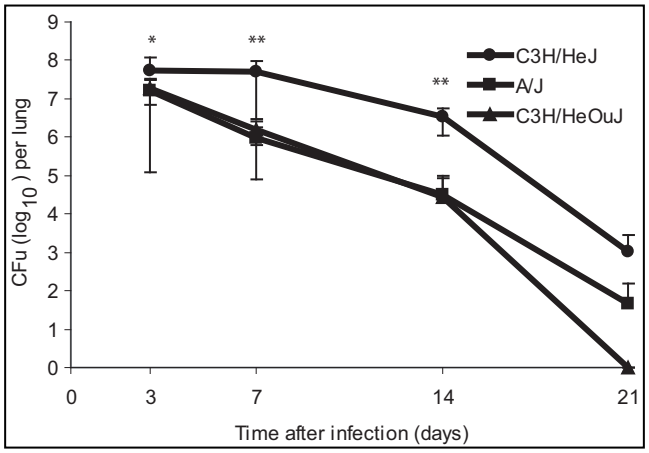

Figure 5: Time course of colonization of B.pertussis in different strains of mice.
CFU's in the lungs of A/J, C3H/HeOuJ (both $Tlr4^{Lps\text{-}n}$) and C3H/HeJ ($Tlr4^{Lps\text{-}d}$) mice. Three, seven, fourteen and twenty-one days after inoculation with B.pertussis, lungs were removed, and the number of viable B.pertussis was determined. The mean of the number of CFU's was plotted with the standard deviation. Asterisks indicate a significant difference in the number of CFU's between C3H/HeJ mice and A/J or C3H/HeOuJ mice. One asterisk indicate a $p<0.05$, 2 asterisks indicate $p<0.01$. The data are representative of two different experiments.

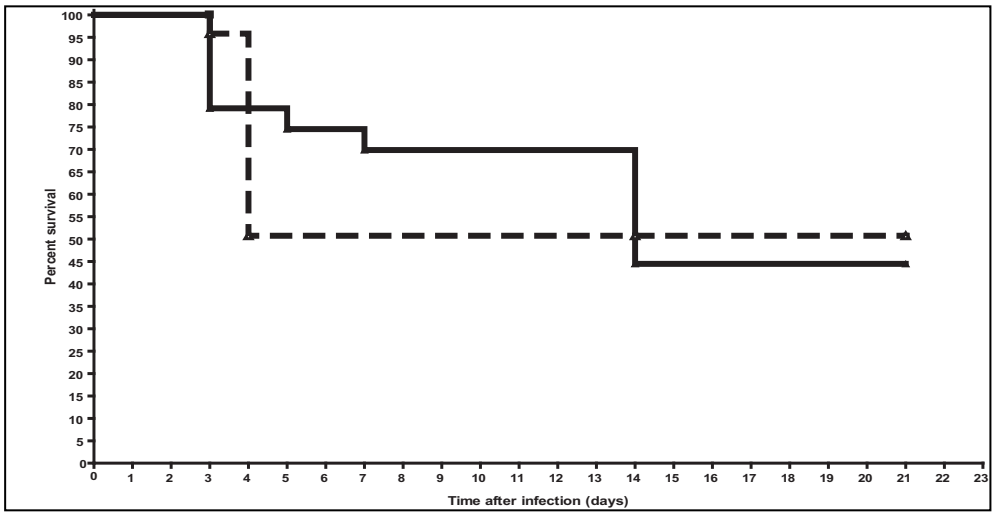

Figure 5: Time course of colonization of B.pertussis in different strains of mice.
CFU's in the lungs of A/J, C3H/HeOuJ (both $Tlr4^{Lps\text{-}n}$) and C3H/HeJ ($Tlr4^{Lps\text{-}d}$) mice. Three, seven, fourteen and twenty-one days after inoculation with B.pertussis, lungs were removed, and the number of viable B.pertussis was determined. The mean of the number of CFU's was plotted with the standard deviation. Asterisks indicate a significant difference in the number of CFU's between C3H/HeJ mice and A/J or C3H/HeOuJ mice. One asterisk indicate a $p<0.05$, 2 asterisks indicate $p<0.01$. The data are representative of two different experiments.

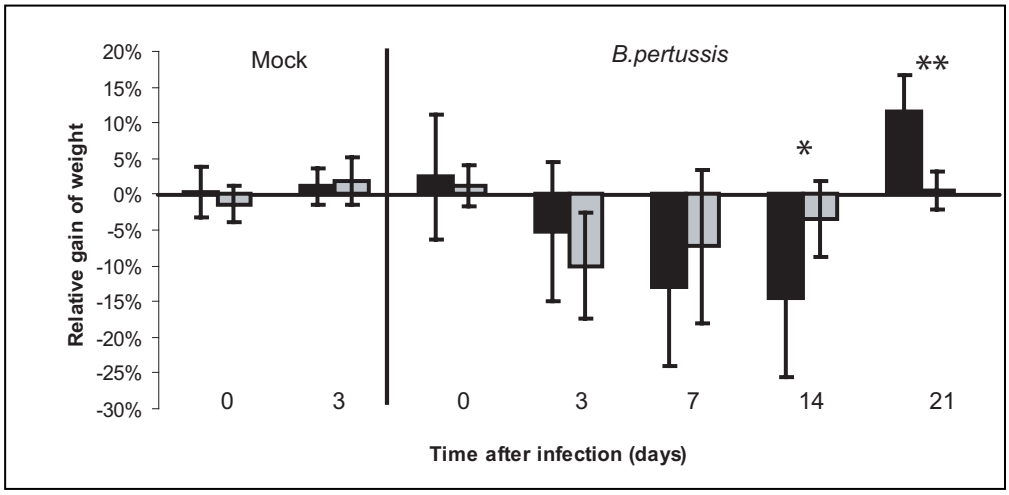

Figure 6: Survival of mice after infection
Survival of mice after infection with B.pertussis. Solid line represents C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$), dashed line represents A/J mice ($Tlr4^{Lps\text{-}n}$).

Lung pathology

The role of *Tlr4* in lung pathology was examined by comparing differences in lung lesions between C3H/HeJ ($Tlr4^{Lps\text{-}d}$) and A/J ($Tlr4^{Lps\text{-}n}$) mice. Lungs were weighed as a marker for inflammation. Relative lung weights are presented in Figure 8. Mock-infected mice had an average relative lung weight of 1%, while *B. pertussis*-infected mice had higher relative lung weights. C3H/HeJ mice had significantly higher relative lung weights after infection compared to A/J mice.

Lung lesions are summarized in Table 2. Examples of typical lesions are presented in Figure 9. The inflammatory response to *B. pertussis* in the two strains of mice examined were similar during the first week after infection. After three and seven days, the peribronchiolar and perivascular inflammation remained slight to moderate. However, two and three weeks after infection, perivasculitis was stronger in the A/J strain than in the C3H/HeJ strain. Also a remarkable difference in the quantity of free protein in the alveoli, which is indicative for inflammatory exudate, was observed. C3H/HeJ mice had a marked inflammatory exudate at three days post infection, which was even more pronounced at days 14 and 21. This inflammatory exudate is very likely responsible for the increased lung weight. In contrast, the A/J mice had no or only a slight inflammatory exudate. No strain differences were seen in peribronchiolitis, alveolitis, the extent of the hypertrophy of the bronchiolar epithelium, and eosinophilia.

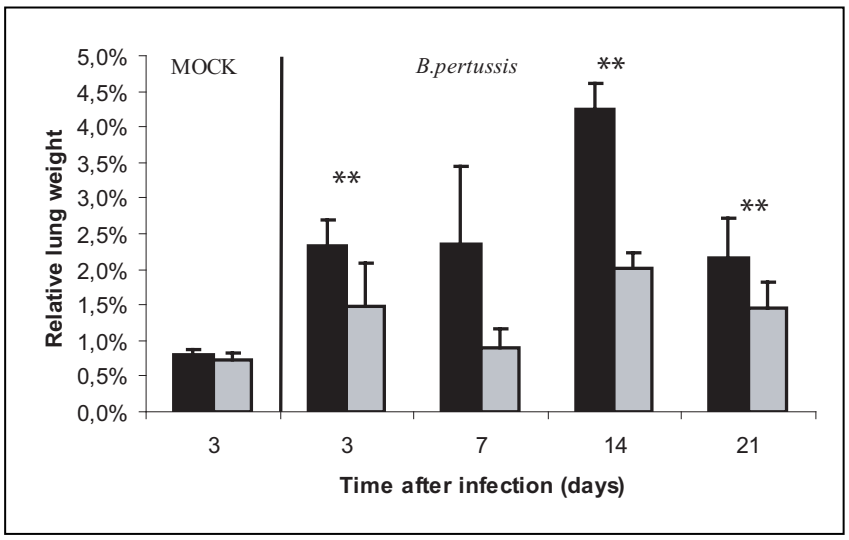

Figure 7: Gain of weight after infection
Gain of weight after B.pertussis inoculation. Solid boxes represent the mean of C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$), grey boxes represent A/J mice ($Tlr4^{Lps\text{-}n}$). Asterisks represent the significance between the two inbred strains at the specific point of time. One asterisk represents $p<0.05$ while two asterisks represent $p<0.001$. The data are representative of two different experiments.

Table 2: Summary of histological lung changes in two strain of mice infected with B. pertussis.

Mouse strain	C3H/HeJ					A/J				
Days after infection	Mock	3	7	14	21	Mock	3	7	14	21
Intercurrent deaths	-	-	2	2	3	-	-	5	2	1
Number examined	6	6	4	4	3	6	6	1	4	5
Peribronchiolitis [a]										
minimal	-	-	-	-	-	-	-	-	-	-
slight	-	6	3	1	-	-	2	1	-	-
moderate	-	-	1	3	3	-	3	-	2	5
marked	-	-	-	-	-	-	-	-	2	-
Perivascular infiltrate										
minimal	3	-	-	-	-	-	-	-	-	-
slight	-	5	-	-	-	-	1	-	-	-
moderate	-	1	4	3	3	-	3	1	-	1
marked	-	-	-	1	-	-	1	-	2	3
strong	-	-	-	-	-		-	-	2	1
Hypertrophy br.epithelium										
minimal	-	-	-	-	-	-	-	-	-	-
slight	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	1	-	2	-	-	1
marked	-	6	1	2	2	-	3	1	1	4
strong	-	-	3	2	-	-	-	-	3	-
Alveolitis										
minimal	-	-	-	-	1	-	-	-	-	2
slight	-	-	-	-	1	-	2	-	-	2
moderate	-	6	2	2	1	-	2	1	-	1
marked	-	-	2	1	-	-	1	-	4	-
strong	-	-	-	1	-	-	-	-	-	-
Protein in alveoli										
minimal	-	-	-	-	-	-	5	-	-	-
slight	-	-	-	1	-	-	-	-	-	-
moderate	-	2	1	1	-	-	-	-	-	-
marked	-	2	-	-	-	-	-	1	-	-
strong	-	2	3	2	-	-	-	-	-	-

[a] *H&E stained slides were examined for peribronchiolitis, alveolitis, perivasculitis, hypertrophy of mucus-producing glands, free protein and eosinophili. Lung lesions were scored semi-quantitatively as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5)*

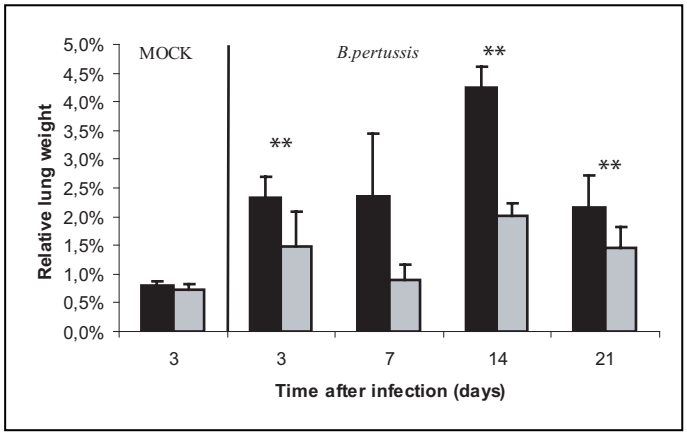

Figure 8: Lung weight after infection
Relative lung weight of mice after infection with B.pertussis. Solid boxes represent the mean of C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$), grey boxes represent A/J mice ($Tlr4^{Lps\text{-}n}$). Asterisks represent the significance between the two inbred strains at the specific point of time. One asterisk represents $p<0.05$ while two asterisks represent $p<0.001$. The data are representative of two different experiments.

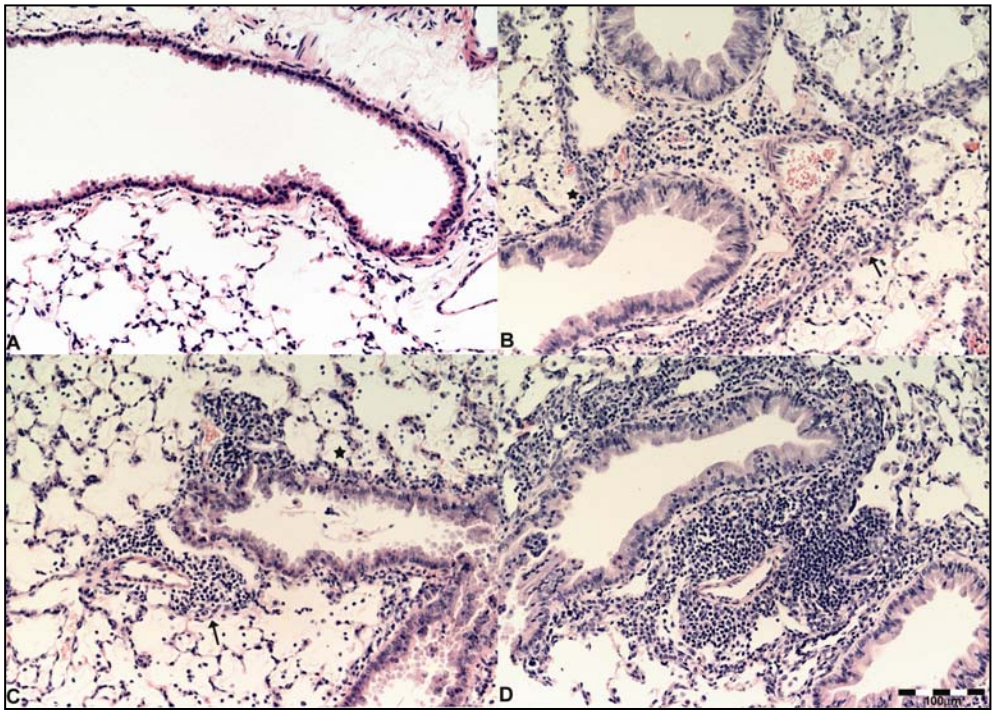

Figure 9: Examples of time dependency of Lung Pathology after infection.
H&E-stained lung sections, bar = 100 µm. ***a.*** *Control A/J mouse bronchiole with thin epithelial layer surrounded by empty alveoli.* ***b.*** *Three days post infection of a C3H/HeJ mouse a slight peribronchiolar (asterisk, score 2) and perivascular infiltrate (arrow) is obvious, together with a moderate hypertrophy of the bronchiolar epithelium (centre right, score 3). The alveoli show a moderate infiltrate and free protein in the alveolar space.*
c. *Seven days post infection a C3H/HeJ mouse shows a slight peribronchiolitis (asterisk) and a moderate perivasculitis (arrow). The hypertrophy of the bronchiolar epithelium is very strong (score 5). In all alveoli free cells as well as protein are present.*
d. *Two weeks after infection an A/J mouse has a moderate peribronchiolitis (score 3) and a marked perivasculitis (centre, score 4). A strong hypertrophy of the bronchiolar epithelium is shown with inflammatory cells inside. Some alveolar macrophages are observed (top, right).*

Cytokine response

IL1-β, TNF-α and IFN-γ production were determined in supernatants of splenocytes isolated at various days after infection from *B. pertussis*-infected A/J and C3H/HeJ mice after 3 days of *ex vivo* re-stimulation with heat-inactivated *B. pertussis* or ConA. Because *Tlr4* engagement also modulates the adaptive Th1 immune response (7,12,18,22), IFN-γ was taken as parameter of the aquired immune response.

As expected, supernatants of cells that were cultured in the absence of antigens did not show cytokine production, while supernatants of cells that were cultured in the presence of ConA showed production of all three cytokines. There was no difference in ConA-induced cytokine production between mouse genotypes or the various days after infection.

The cytokine production of splenocytes restimulated with heat-inactivated *B. pertussis* is shown in Figure 10. Culture supernatants from splenocytes isolated from A/J mice showed an increase of IL1-β, TNF-α and IFN-γ from 3 days till 14 after infection. At day 21 after infection, the cytokine concentrations decreased. In contrast, culture supernatants from splenocytes isolated from C3H/HeJ mice only showed a small cytokine response. No IL1-β response and only a slight increase in TNF-α and IFN-γ production was seen from 14 days after infection onwards.

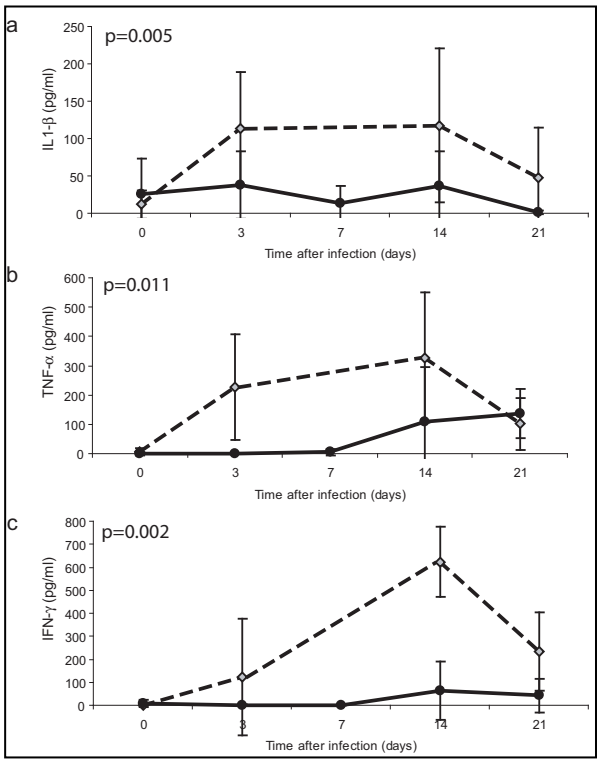

Figure 10: Cytokine production after ex vivo stimulation of splenocytes

IL1-β (a), TNF-α (b) and IFN-γ (c) cytokine production of splenocytes isolated from B.pertussis-infected mice after 3 days of ex vivo re-stimulation with heat-inactivated B.pertussis. Solid line represents the mean of C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$), dashed line represents A/J mice ($Tlr4^{Lps\text{-}n}$). The plotted p-value represents the overall difference in cytokine production between the mice.

TLR4 and TNF-α gene expression

The Tlr4-dependend induction of TNF-α has been shown to be critical for the early host response to *B.bronchiseptica* (20,21). Therefore, we examined gene expression of *Tlr4* and TNF-α in lung tissue of mice three days after infection with *B. pertussis*. Data are presented in Figure 11 as fold expression relative to mock-treated A/J mice (shown in grey). All expression data are calculated relative to the housekeeping gene RNA polymerase IIa (Polr2a). C3H/HeJ mice, either MOCK-treated or *B. pertussis* infected, showed no upregulation of expression of *Tlr4,* while *B. pertussis*-infected A/J mice showed a 1.5-fold upregulation of this receptor. In addition *B. pertussis*-infected A/J mice showed a 5-fold upregulation of TNF-α, while infected C3H/HeJ mice only showed a slight, insignificant upregulation in expression of this gene.

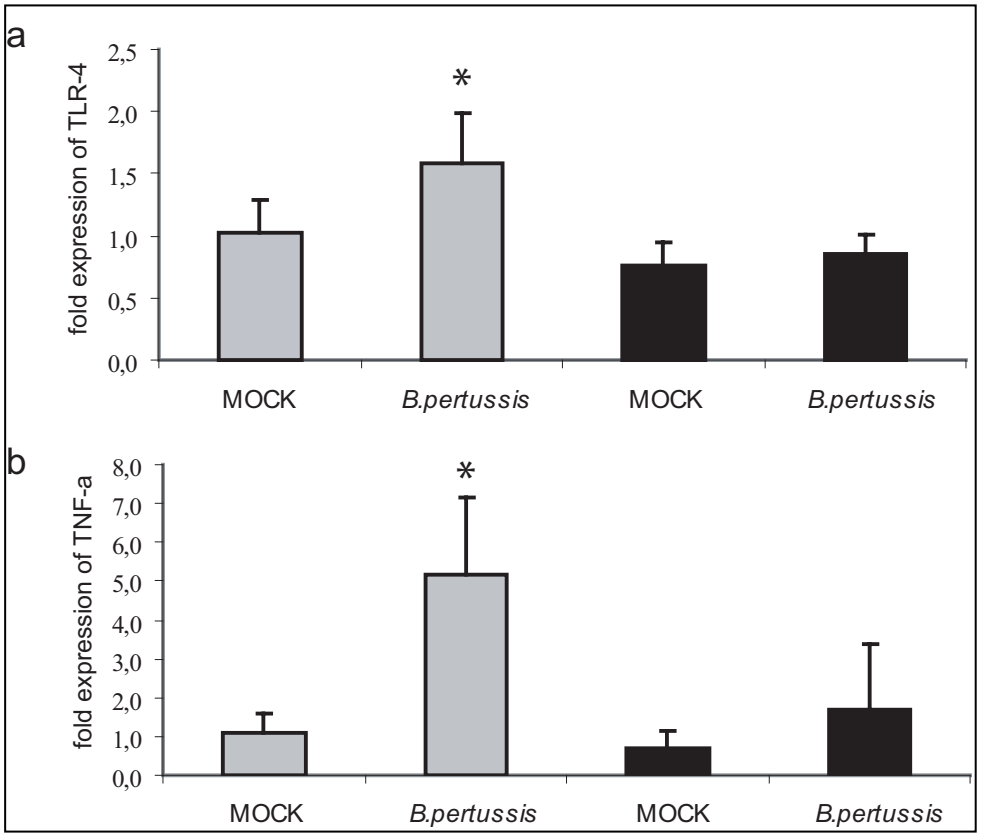

Figure 11: Gene expression of TLR-4 and TNF-α in infected lung tissue.
Gene expression of TLR-4 (a) and TNF-α (b) in lung tissue of mice three days after inoculation with B.pertussis. Gene expression was compared relative to mock-treated A/J mice. Solid boxes represent the mean of 5 C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$), grey boxes represent the mean of 5 A/J mice ($Tlr4^{Lps\text{-}n}$). Stars indicate a significant difference of $p<0.05$ according to the Bonferoni test between groups.

Discussion

By previously identifying three susceptibility loci (*Bps1 – 3)* in recombinant congenic strains of mice we showed that the clearance and/or replication of pertussis in mice is under multigenic control. These loci could explain up to 10% of the variation in the lung colonization observed in F2 mice (2). Because the contribution of these loci to genetic variation was small, we started the present study using inbred strains of mice instead of recombinant congenic strains of mice. By using this approach, we expected to examine a broader range of genetic variation, possibly leading to the identification of additional susceptibility loci.

Significant differences in clearance were observed between these strains of mice. From these strains we selected A/J as the most resistant strain and C3H/HeJ as the most susceptible strain, to generate an F2 intercross generation and to subsequently identify susceptibility loci, and to examine phenotypic characteristics.

We had originally planned to use a low-density genome-wide scan, with SNP markers, but mainly due to the Poltorak paper(26) we started by analyzing this particular mutation in Tlr4. It turned out that the association between CFU's and this mutation was so prominent that we calculated that possible other genetic effects could not be detected in this F2 generation. We therefore have not performed a whole genome-wide scan, but concentrated on markers on chromosome 4 as displayed in Figure 2.

We subsequently identified Tlr4 as a major factor that influences the course of *B. pertussis* infection in mice. We calculated by linkage analysis that a large part of chromosome 4 was linked with reduced clearance of bacteria from the lungs , with a maximum LOD-score of 33.6 at 65.4Mb, which is the location of *Tlr4*.

As the number of mice was too large for analysis on a single day, the experiments were executed on multiple days. Because BALB/c control mice were included in each of these days we were able to estimate the contribution of *Tlr4* to the total variation. We calculated that the residual mean square error of the BALB/c mice is larger than the residual mean square error of the *Tlr4* genotype. This means that the residual variation is smaller than the variation in inbred strains, strongly suggesting that no additional detectable genetic variation was left. We therefore concluded that the residual variation is due to environmental variation, and that all of the genetically described variation in these experiments could be ascribed to the *Tlr4* gene. Although other (modifier) genes are expected to influence the course of pertussis, it is not possible to detect the effect of these genes in F2 hybrids generated from these two strains of mice due to the strong effect of *Tlr4*. We did, however, analyze markers located in *Bps1,* but did not find any linkage, probably due to the reason mentioned before. Thus, *Tlr4* dominates the variation in clearance of bacteria from the lungs in the first week after infection in these strains of mice.

To confirm the importance of *Tlr4* function in *B. pertussis* infection, we infected mice of A/J(*Tlr4*$^{Lps\text{-}n}$), C3H/HeOuJ(*Tlr4*$^{Lps\text{-}n}$) and C3H/HeJ (*Tlr4*$^{Lps\text{-}d}$). C3H/HeOuJ mice are genetically identical to C3H/HeJ mice except for the pro712his mutation in the gene encoding for *Tlr4* (31). A/J and C3H/HeOuJ mice showed the same course of clearance of bacteria from the lung, while C3H/HeJ mice showed a delayed clearance of bacteria from the lung. Thus this experiment unequivocally confirmed the significance of Tlr4.

Remarkably, although A/J mice cleared *B. pertussis* more efficiently, we observed no difference in overall mortality after *B. pertussis* infection between A/J and C3H/HeJ mice. However A/J mice died earlier than C3H/HeJ mice. Thus mortality appeared associated in time with the inflammatory response. As described below, additional inflammatatory and pathobiological parameters confirmed the association between bacterial clearance, inflammation and clinical effects.

In A/J mice, cytokine production of splenocytes re-stimulated *ex-vivo* with heat-inactivated *B. pertussis* showed an increase of IL1-β, TNF-α, and IFN-γ production, three days after infection, with a maximum at day 14 after infection. In C3H/HeJ mice, however, there was very little production of IL1-β, and little production of TNF-α, and no IFN-γ production. Although Higgins et.al. (11) suggested a role for Tlr4 in inhibiting Th1 responses by activating IL-10 production, our results indicate that *Tlr4* engagement is not only essential for the innate response, but also for stimulation of the ensuing Th1 response, which is in line with other reports (7,12,18,22). Functional Tlr4 may be required for the production of IL-10, which is associated with limiting the inflammatory pathology (11). In the present study we observed enhanced lung pathology in Tlr4-defective mice, especially enhanced lung weight, which underlines the significance of Tlr4 limiting bacterial growth and probably also inflammation. However, it is likely that in C3H/HeJ mice pathology resulting from infection differs from that in TLR-4 competent mice as a result of different levels of expression or production of IL-1β, TNF-α, and IFN-γ. In response to LPS inhalation a TNF-α-dependent neutrophil influx in the BALF is seen in C3H/HeN but not in C3H/HeJ mice (10). In addition, gene profiling of lung tissue showed that 74% of the genes responsive to a *Klebsiella pneumoniae* infection was TLR-4 dependent(28). Interestingly, during lung inflammation C3H/HeJ mice did not show perivascular accumulation of inflammatory cells, whereas A/J mice did (29). In addition, *Escherichia coli* inhalation resulted in a similar clearance in C3H/HeJ and in C3H/HeSnJ mice, but resulted in lower proinflammatory cytokine levels and reduced neutrophils accumulation in C3H/HeJ mice(17).

Three days after infection *B. pertussis*-infected C3H/HeJ mice showed no expression of *Tlr4* while similarly treated A/J mice showed a 1.5-fold upregulation of this receptor. This upregulation may be suggestive of actual receptor engagement. *B. pertussis*-infected A/J mice showed a 5-fold upregulation of TNF-α, while C3H/HeJ mice only showed a slight, not significant,

upregulation of this gene. This suggests that Tlr4-dependent TNF-α expression in the lung is an innate response to *B. pertussis* infection, similar to the observations for *B. bronchiseptica* (20,21). It has been shown that early elicited TNF-α release is critical for host defence against *B. bronchiseptica,* and that this process is Tlr4 dependent (20,21). Although the course of infection with *B. bronchiseptica* is different from infection with *B. pertussis,* our data underline the significance of early TNF-α production in limiting bacterial growth. In C3H/HeJ mice other, possibly compensatory mechanisms might be operational. Functional Tlr4 seems to affect TNF-α levels both in lung and spleen.
Also in humans the role of *Tlr4* in infectious diseases has been investigated in genetic association studies (27). Associations were usually investigated for two SNP's, Asp299Gly and Thr399Ile, that result in amino acid changes in the extracellular domain of the receptor. Associations were found in some reports, but not in others, as recently reviewed by Schröder and Schumann (27).
In conclusion, we have shown that C3H/HeJ mice have delayed clearance of bacteria from the lung, increased lung pathology, more weight loss, and a lower IL1-β, TNF-α, and IFN-γ production after infection compared to A/J mice. We showed that *Tlr4* is a major genetic factor that was sufficient to explain all detectable genetic differences in bacterial clearance between these mice. Functional Tlr4 is required for an early TNF-α, IL1-β, and IFN-γ response that may enhance bacterial clearance and thus, despite the proinflammatory nature of theses cytokines may limit pathology. This early cytokine response was fatal for app. 50% of the mice, but the surviving mice had efficiently cleared the bacteria from the lungs, and little lung pathology and rather quickly regained their body weight. A lack of this response resulted in delayed mortality, but these mice were less efficient in clearing the bacteria from the lungs and became very ill from lung edema, slowly regained their body weights.

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Chapter 6

The role of Toll-like receptor-4 in pertussis vaccine-induced immunity.

Submitted for publication

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Abstract
Pertussis is an infectious disease of the respiratory tract. The gram-negative bacterium *Bordetella pertussis* is an important causative agent of this disease. In most developed countries whole-cell pertussis vaccines (wP) have been replaced by acellular vaccines (aP), because wP were found to be reactogenic. In contrast to wP, aP do not contain LPS, raising concerns on the efficacy of aP, and also on the efficacy of vaccines administered simultaneously with aP. Adjuvants like LPS that activate toll-like receptor-4 (Tlr4) may improve the efficacy of bacterial subunit vaccines. To establish the role of Tlr4 in pertussis vaccine-induced immune responses we compare *Tlr4*-deficient C3H/HeJ and wild-type C3H/HeOuJ mice in our model of *B. pertussis* challenge after wP or aP vaccination. aP consisted of pertussis toxin (Ptx), filamentous hemagglutinin (FHA), and pertactin (Prn).
In *Tlr4*-deficient mice, compared to wild-type controls (i) after vaccination only, Ptx-IgG, FHA-IgG, and Prn-IgG levels were similar, (ii) after infection (only), the number of bacteria was higher, while IL-1α and IL-1β expression and IFN-γ and IL-17 production were lower, (iii) after wP vaccination and challenge, the number of bacteria, Prn-IgG levels, and IL-5 expression were higher, while IL-1β, TNF-α, IFN-γ, IL-17, and IL-23 expression and IL-17 production were lower, and lung pathology was absent, and (iv) after aP vaccination and challenge, the number of bacteria, Prn-IgG levels, and IL-5 expression were higher, while Ptx-IgG levels were lower.
Tlr4 plays an important role in natural immunity, wP and aP efficacy, and the induction of Th1 and Th17 responses after wP vaccination and challenge. Tlr4 affects a more limited set of parameters in case of aP vaccination than in case of wP vaccination. The presence of functional Tlr4 is critical for lung pathology after wP vaccination and challenge, enhances pro-inflammatory cytokine production after wP vaccination and challenge, and diminishes Th2 responses after wP and aP vaccination and challenge. The humoral response to vaccination (without challenge) is not influenced by Tlr4. The importance of Tlr4 in efficacy underlines a role for Tlr4 agonists (such as LPS analogs) in improving efficacy of bacterial subunit vaccines.

Background

Pertussis is among the ten infectious diseases with the highest morbidity and mortality worldwide. An important causative agent of pertussis is the gram-negative bacterium *Bordetella pertussis.* After introduction of whole-cell vaccines (wP) in the 1950's, pertussis incidence has decreased significantly. Although being efficacious, wP vaccines were found to be reactogenic. Therefore, acellular vaccines (aP) comprising purified *B. pertussis* proteins have been developed. Concerns have been raised, however, with respect to the relative efficacy of aP as compared with wP, and also of vaccines administered simultaneously with aP, such as diphtheria, tetanus, polio, and *Haemophilus influenzae* b (Hib) vaccines (1).

It has been suggested that the limited efficacy of aP may be caused, in part, by the fact that, in contrast to wP, aP do not contain lipopolysaccharide (LPS). In mice Th17 cells (2) as well as Th1 cells are induced by wP vaccination, but not (or to a much lesser extent) by aP vaccination (3). Furthermore, the induction of these Th17 and Th1 cells is Tlr4-dependent (3). Importantly, Th17 and Th1 cells are critical for clearing a *B. pertussis* challenge (3). Together, these results underline the importance of Tlr4 (and hence, also its agonists such as LPS) in generating immune responses induced by pertussis vaccines, at least in mice. Besides, Tlr4 is also critical for pertussis clearance and ensuing adaptive immunity in non-vaccinated mice (4,5,6). The efficacy of vaccines that do not contain LPS, such as viral vaccines, can be improved by supplementation with Tlr4 agonists that show no or little endotoxin activity. We have shown that supplementing the bacterial subunit vaccine aP with the Tlr4 agonist monophosphoryl lipid A improved its efficacy (7).

We have previously shown that Tlr4 influenced lung pathology and production of proinflammatory cytokines, such as IL-1β and TNF-α, after *B. pertussis* infection of mice (9). In another study, we have shown that (particularly) wP vaccination and *B. pertussis* challenge induced lung pathology and lung TNF-α expression (8). In addition, wP and especially aP vaccination and *B. pertussis* challenge induced type I hypersensitivity, a Th2 driven response (8). A role for Tlr4 was, however, not addressed. To improve our understanding of supplementing aP with Tlr4 agonists, and following data from our group (6,8) and others (3,4,5) we studied the role of Tlr4 in bacterial clearance, humoral immune response, and lung pathology. To detect inflammatory as well as Th1, Th17, and Th2 responses, lung and systemic cytokine expression was measured.

In this study, we confirm that Tlr4 plays an important role in natural immunity, and show its important role in wP and aP efficacy, as well as induction of Th1 and Th17 responses. The set of parameters affected by Tlr4 is more limited in case of aP vaccination than in case of wP vaccination. The presence of functional Tlr4 is critical for lung pathology after wP vaccination and challenge, enhances pro-inflammatory cytokine production after wP vaccination and challenge, and diminishes Th2 responses after both wP and aP vaccination and challenge. The humoral response to vaccination (without challenge) is not influenced by Tlr4.

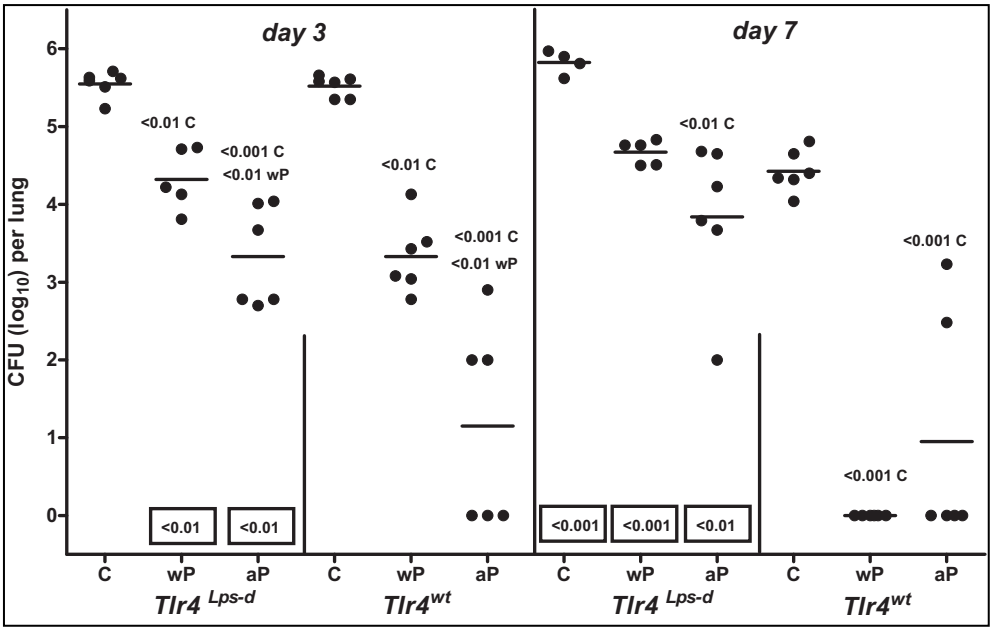

Figure 1: Colonization of the lungs by B. pertussis.
Tlr4-deficient C3H/HeJ and control C3H/HeOuJ mice were subcutaneously (sc) injected with 1/5 human dose (HD) wP, aP, or adjuvant (C), twice before intranasal B. pertussis infection. Three and seven days after challenge lungs were excised, and the number of viable B. pertussis was determined in right lung lobes. Each symbol represents the number of bacteria in the lung of an individual mouse; horizontal lines represent the group average. (**) P<0.01; (***) P<0.001. Non-boxed asterisks show the P-value against the indicated group. Boxed asterisks show the P-value against the wild-type mouse strain. ANOVA followed by Bonferroni post-hoc test. A single representative experiment of 2 is shown.

Materials and Methods

Animals

Female mice were used at 6-8 weeks of age. $Tlr4^{Lps\text{-}d}$ C3H/HeJ (31) and wild-type control C3H/HeOuJ mice were obtained from Jackson (Bar Harbor, ME). The diet consisted of ground standard laboratory chow (RMH-B, Hope Farms, Woerden, the Netherlands). Food and water were given ad libitum. All animal experiments were performed according to national and international guidelines.

Vaccines

The wP vaccine is a whole-cell pertussis vaccine, combined with vaccines against diphtheria, tetanus, and poliomyelitis, and produced by the Netherlands Vaccine Institute, Bilthoven, the Netherlands. One human dose (HD) contains $1.6x10^{10}$ CFU of two *B. pertussis* strains (strains 509 and 134; 8 opacity units /strain) in 1 ml saline, and is adjuvated with 1.5 mg aluminum phosphate.

The aP vaccine is 3-component acellular pertussis vaccine, combined with vaccines against diphtheria and tetanus, and produced by GlaxoSmithKline, Rixensart, Belgium. One HD contains 25 μg formaldehyde- and glutaraldehyde-detoxified pertussis toxin, 25 μg filamentous hemagglutinin, and 8 μg pertactin in 0.5 ml saline, and is adjuvated with aluminum hydroxide (< 0.625 mg Al per HD).

Vaccination

One HD wP or aP was diluted with 0.5% $Al(OH)_3$ gel (Serva, Heidelberg, Germany) to a final volume of 2.5 ml. Mice (6 per group) received a subcutaneous injection with 0.5 ml of 1/5 HD wP, 1/5 HD aP, or adjuvant alone, 28 and 14 days before infection (8,11). Note that the ratio between adjuvant and bacteria (in case of wP) or between adjuvant and proteins (in case of aP) was considerably higher in the murine vaccine doses than in the original human vaccine doses.

Infection of mice and autopsy

The mice were anaesthetized with isoflurane. Two h before infection, 3 drops of blood were collected from the orbital plexus. A single drop of 40-μl inoculum containing $2x10^7$ *B. pertussis* cells (Tohama strain B213) was carefully placed on the top of the nose and allowed to be inhaled ((32)).

Mice were sacrificed 3 or 7 days after challenge, except in case of lung pathology in which case the mice were sacrificed 3 days after infection only. They were anaesthetized with ketamine, rompun, and atropine, and blood was

collected from the orbital plexus. Perfusion of the right ventricle was performed with 2 ml PBS supplemented with 3.5% heat-inactivated Fetal Calf Serum (FCS; PAA, Linz, Austria). The lungs were excised and used to obtain bronchial lymph nodes (LN) and lung lobes. The left lung lobes were formalin-fixed for histological examination or collected in RNA-later (Qiagen, Venlo, the Netherlands) for RNA extraction, while the right lung lobes were used for enumeration of bacteria.

Lung lobes, CFU determination, and histological examination

A ligature was made around the right bronchus after which the right lobes were removed for enumeration of bacteria. The lobes were homogenized in 900 μl of Verwey medium using a tissue homogenizer (Pro-200, ProScientific, Monroe, CT) at maximum speed for 10 s. The homogenates were diluted in Verwey medium 10- and 100-fold for the immunized mice, and 1000-fold for the control mice. Hundred-μl aliquots of the dilutions were plated on BG plates supplemented with streptomycin and incubated at 35°C for 5 days. The remaining left lung lobes were fixed intratracheally using 4% formalin for 24 h. After overnight dehydration, they were embedded in paraffin. Five-μm sections were cut and stained with haematoxylin/eosin. Histological lesions were semi-quantitatively scored as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. This score incorporates the frequency as well as the severity of the lesions.

Ptx-, FHA-, and Prn-specific IgG

Pertussis toxin (Ptx) and filamentous hemagglutinin (FHA) were obtained from Kaketsuken, Kumamoto, Japan. Pertactin (Prn1) was prepared as described previously (33). Immulon 2HB plates (Thermo Scientific, Waltham, MA) were coated with 2 μg/ml Ptx, 2 μg/ml FHA, or 5 μg/ml Prn1, in PBS, incubated overnight, and washed. After addition of a dilution series of control and test sera, the plates were incubated for 1 h and washed. After addition of detection antibody (1:5,000 anti-mouse IgG-horseradish peroxidase (HRP); Southern Biotechnology Associates, Birmingham, AL) in PBS, 0.1% Tween-80, 0.5% Protifar (Nutricia, Zoetermeer, the Netherlands), the plates were incubated for 1 h and washed. To detect HRP, the plates were washed and incubated for 10 min in 10% sodium acetate, 1% tetramethylbenzidine (Sigma, Axel, the Netherlands), and 0.01% H_2O_2. 2M H_2SO_4 was added to stop color development and the plates were read at 450 nm. Washing steps were 5 times with PBS-0.03% Tween-80. Incubations were at room temperature.

Cell culture

The culture medium used was RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Cell suspensions were made by pressing the LN or spleens through a cell strainer (Falcon, Franklin Lakes, NJ). Cells were counted using a Coulter Counter (Coulter Electronics, Luton,

UK). LN cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Concanavalin A (Con A; MP Biomedicals, Irvine, CA) in flat-bottom 12-well culture plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Spleen cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Con A or *B. pertussis* (10^5 heat-inactivated bacteria per well) in 96-well tissue culture plates (Nunc) at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Bacteria were heat-inactivated at 56°C during 30 min.

Cytokine measurements

An 8-plex panel containing beads for mouse IL-1α, IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ, and TNF-α (Bio-Rad, Hercules, CA) was used. After incubation and washing steps (see (8) for details) the beads were measured on a Bio-Plex (Bio-Rad).

Cytokine expression

Cytokine mRNA expression was measured using Taqman gene expression assays (Applied Biosystems, Foster City, CA) as described previously (6,8). Briefly, RNA was extracted with an RNeasy kit (Qiagen). Copy DNA was generated using the High Capacity cDNA archive kit containing random hexamer primers (Applied Biosystems). Messenger RNA expression was measured on a 7500 Fast Real-Time PCR System (Applied Biosystems). We used the assay on demand (Applied Biosystems) for IL-1α (Mm99999060), IL-1β (Mm01336189), IL-4 (Mm00445259), IL-5 (Mm00439646), IL-17 (Mm00439618), IL-23 (Mm00518984), IFN-γ (Mm00801778), and TNF-α (Mm00443258). For the reference gene, hypoxanthine phosphoribosyl transferase (HPRT), the assay was designed using the primer express program (Applied Biosystems) resulting in probe CAGTCCTGTCCATAATCA, forward primer GCCGAGGATTTGGAAAAAGTGTTTA, and reverse primer TTCATGACATCTCGAGCAAGTCTTT. The relative concentration of the various mRNA's was determined by the comparative threshold cycle method (ddCt) (34-36).

The fold change in mRNA expression is relative to lung tissue of untreated C57BL/6J mice. This third-party control strain does not favor either of the two strains of mice tested (the C3H/HeJ or the C3H/HeOuJ strain).

Statistics

One-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test was performed (SPSS, Chicago, IL). Histological data were analyzed using the non-parametric Mann-Whitney U test (SPSS).

Results

B. pertussis colonization of the lungs

Tlr4-deficient C3H/HeJ and wild-type control C3H/HeOuJ mice (6 per group) were vaccinated twice with 1/5 human dose (HD) wP, 1/5 HD aP, or adjuvant, and challenged intranasally with *B. pertussis*. Three and seven days after infection, mice were sacrificed and CFU in the lungs were enumerated. Vaccinated mice generally showed a lower number of bacteria than adjuvant-treated animals, while *Tlr4*-deficient mice generally showed a higher number of bacteria than wild-type animals (Figure 1). Adjuvant-treated *Tlr4*-deficient mice showed an increasing number of bacteria from day 3 till day 7 (Δ log CFU = 0.28, P = 0.029), while similarly treated wild-type animals showed a decreasing number in this time period (Δ log CFU = -1.09, $P < 0.001$). *Tlr4*-deficient mice that were wP-vaccinated before challenge failed to show a decreasing number of bacteria from day 3 till day 7, while similarly treated wild-type animals did show a decreasing number in this time period (Δ log CFU = -3.33, $P < 0.001$). In aP-vaccinated mice of either strain the number of bacteria was similar between day 3 and day 7.
These data show (i) the important role of Tlr4 in the natural defense to *B. pertussis* infection and confirms our earlier findings (6), (ii) the important role of Tlr4 in wP-induced clearance, and (iii) vaccine-induced clearance is faster after aP vaccination than after wP vaccination, regardless of the presence of Tlr4. This latter notion is supported by the higher clearance in aP-vaccinated than wP-vaccinated mice at day 3 but not day 7 in either mouse strain.

Vaccination-induced protection, i.e. the difference in the number of bacteria between vaccinated and control mice, was lower in *Tlr4*-deficient mice than in wild-type animals ($P < 0.01$), except for aP vaccination at day 7 where this effect was not statistically significant (Δ log CFU = -1.23 and -2.19 (day 3, wP), -2.22 and -4.37 (day 3, aP), -1.15 and -4.43 (day 7, wP), and -1.99 and -3.48 (day 7, aP) for *Tlr4*-deficient and wild-type mice, respectively).
Collectively, these data show that Tlr4 is not only involved in the natural defense to *B. pertussis* infection but also in vaccination-induced protection to this pathogen, both after wP and after aP vaccination.

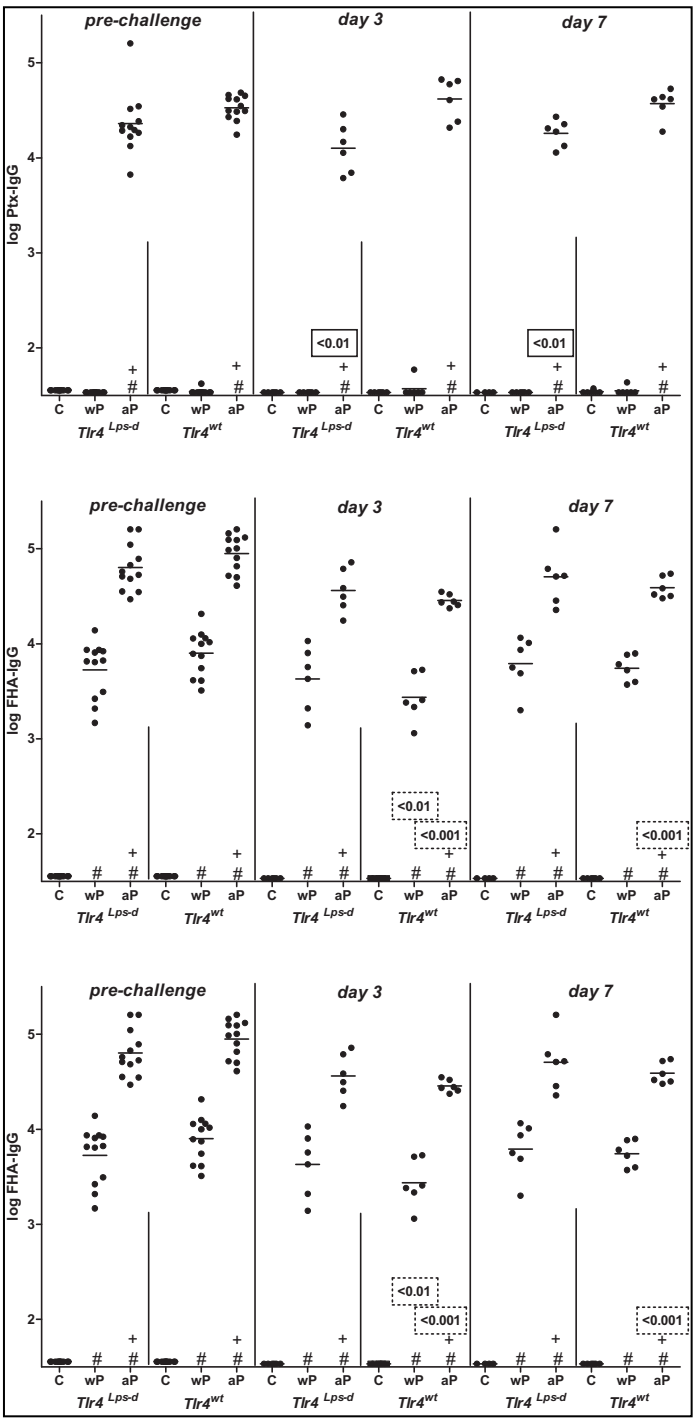

Figure 2: Ptx- (A), FHA- (B), and Prn- (C) specific IgG in serum.
Tlr4-deficient and control mice were sc injected with 1/5 HD wP, aP, or adjuvant (C), twice before intranasal B. pertussis infection. Two hours before challenge, and three and seven days after challenge blood was taken. Test and positive control sera were tested for Ptx-, FHA-, and Prn-specific IgG. Each symbol represents the serum level of an individual mouse; horizontal lines represent the group average. (#) increased compared to its adjuvant-treated control ($P < 0.001$), (+) increased compared to corresponding wP-vaccinated mice ($P < 0.001$). (*) $P<0.05$, (**) $P<0.01$, and (***) $P<0.001$. Non-boxed asterisks show the P-value against the indicated group, boxed asterisks show the P-value against the wild-type mouse strain, and stippled-boxed asterisks show the P-value against the pre-challenge levels. ANOVA followed by Bonferroni post-hoc test. A single representative experiment of 2 is shown.

Ptx-, FHA-, and Prn-specific IgG
Serum was taken 2 hours before challenge and 3 and 7 days after challenge, and analyzed for IgG specific for Pertussis toxin (Ptx), filamentous hemagglutinin (FHA), and Pertactin 1 (Prn). Only aP-vaccinated mice showed significant Ptx-IgG levels. Before challenge these levels were not affected by Tlr4 (Figure 2A). At days 3 and 7 after challenge *Tlr4*-deficient aP-vaccinated mice showed significantly lower Ptx-IgG levels than similarly treated wild-type animals. In each mouse strain Ptx-IgG levels were similar before and after challenge.

Both wP- and aP-vaccinated mice showed significant FHA-IgG levels. aP-vaccinated mice showed higher levels than wP-vaccinated animals (irrespective of challenge; Figure 2B). Both before and after challenge FHA-IgG levels were not affected by Tlr4. Generally, both wP- and aP-vaccinated wild-type mice but not *Tlr4*-deficient animals showed lower FHA-IgG levels after challenge than before.

Both wP- and aP-vaccinated mice showed significant Prn-IgG levels (Figure 2C). Generally, aP-vaccinated mice showed higher Prn-IgG levels than wP-vaccinated animals. Before challenge Prn-IgG levels were not affected by Tlr4. Rather unexpectedly after challenge Prn-IgG levels were higher in wP- and aP-vaccinated *Tlr4*-deficient mice than in similarly treated wild-type animals.

Collectively, these data show that before challenge Tlr4 does not affect vaccination-induced Ptx-, FHA-, and Prn-IgG levels. After challenge, Ptx-IgG levels are lower in *Tlr4*-deficient mice than in wild-type animals, while the reverse is true for Prn-IgG levels. FHA-IgG levels are unaffected by the *Tlr4* mutation.

Weight gain
Mice were weighed immediately before challenge, and 3 and 7 days after challenge (Figure 3). Irrespective of the animal strain, in this time period the adjuvant-treated and challenged mice lost weight, while both wP- and aP-vaccinated and challenged animals gained weight. Only aP-vaccinated mice at day 7 after challenge showed a weight gain that was significantly higher in wild-type mice than in *Tlr4*-deficient animals.
Taken together, these data show that vaccination but not (or to a much lesser extent) Tlr4 affects weight gain during the first 7 days after *B. pertussis* challenge.

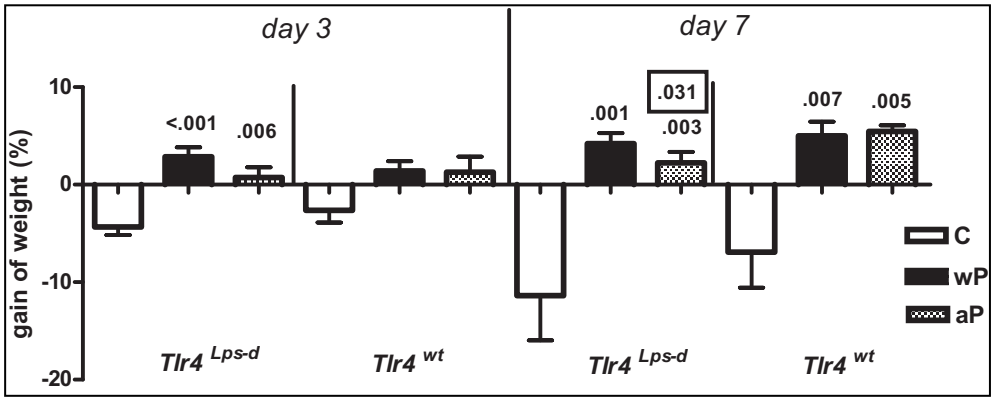

Figure 3: Gain of weight.
Tlr4-deficient and control mice were sc injected with 1/5 HD wP, aP, or adjuvant (C), twice before intranasal B. pertussis infection. Two hours before challenge, and three and seven days after challenge mice were weighed. Data are indicated as mean ± SEM (N=6). Non-boxed numbers show the P-value against the adjuvant-treated and challenged group. The boxed number shows the P-value against the wild-type strain. ANOVA followed by Bonferroni post-hoc test. A single representative experiment of 2 is shown.

Histological changes

We have previously shown that after challenge wP- and aP-vaccinated mice revealed increased lung pathology compared to animals that had only received adjuvant, with wP-vaccinated mice showing more severe pathology than aP-vaccinated animals (8). Here we addressed whether Tlr4 influenced this pathology.

Lung lesions were scored 3 days after challenge (Figure 4). No effect of Tlr4 on lung pathology was seen after challenge of adjuvant-treated mice. In wild-type mice, wP but not aP vaccination resulted in a more severe challenge-induced peribronchiolitis, perivasculitis, and alveolitis compared to adjuvant treatment and challenge, rather similar to our previous findings (8). Lung pathology was less severe in similarly treated *Tlr4*-deficient mice. Vaccination resulted in less severe challenge-induced alveolitis compared to adjuvant treatment, in wild-type mice for aP vaccination and in *Tlr4*-deficient animals for both wP and aP vaccination. No treatment effects on hypertrophy of the bronchiolar mucous cells were seen in either strain, while eosinophilia was absent in all groups.

These data show that challenge-induced pathology in wP-vaccinated animals is Tlr4-dependent.

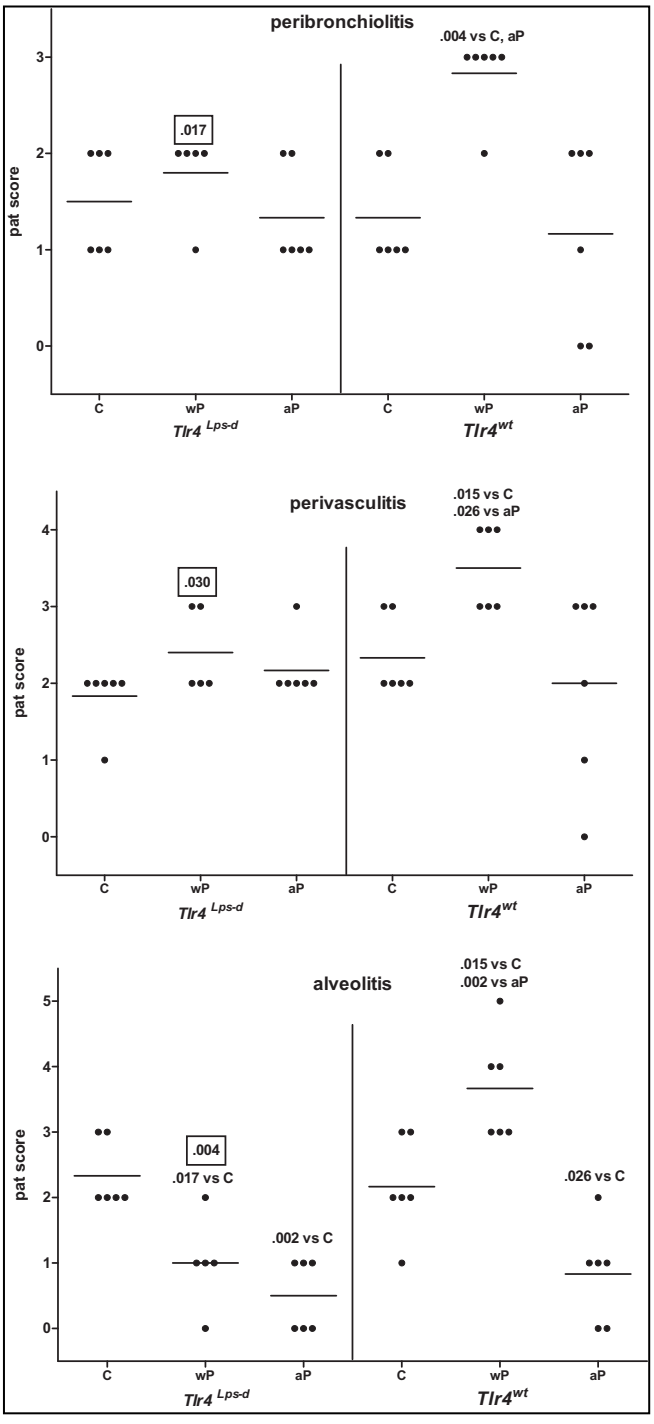

Figure 4: Lung pathology. *Tlr4-deficient and control mice were sc injected with 1/5 HD wP, aP, or adjuvant (C), twice before intranasal B. pertussis infection. Three days after infection the left lung lobes were excised. H&E staining. Histological lesions were semi-quantitatively scored as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. Each symbol represents an individual mouse; horizontal lines represent the group average. Non-boxed numbers show the P-value against the indicated group. Boxed numbers show the P-value against the wild-type strain. Mann-Whitney U test. A single representative experiment of 2 is shown.*

Cytokine gene expression in lung tissue

In this analysis we focused on (i) proinflammatory cytokines, as *B. pertussis* infection induced the production of TNF-α, IL-6, and IL-1β in a Tlr4-dependent way (4,5,6), (ii) Th1 and Th17 cytokines, as Th1 and Th17 cells were induced by wP vaccination in a Tlr4-dependent way and they were shown to be critical for clearance after wP-vaccination (3), and (iii) Th2 cytokines, as pertussis vaccination (especially aP) before challenge induced a Th2-dependent response (8), and this response could be skewed towards a Th1 response by adding LPS analogs (7). Post-challenge lung lobes were subjected to real-time PCR. The fold change is relative to lung tissue of untreated C57BL/6J mice. Irrespective of prior vaccination, IL-1α mRNA expression is lower in challenged C3H mice than in untreated C57BL/6 mice, while the reverse is true for the other cytokines analyzed.

Proinflammatory cytokines. Tlr4-deficient adjuvant-treated mice showed higher IL-1α and IL-1β expression than similarly treated wild-type animals at day 7 after challenge (Figure 5A). *Tlr4*-deficient wP-vaccinated mice revealed lower IL-1β and TNF-α expression than similarly treated wild-type animals at day 3 after challenge. In general, in both strains of mice both wP and aP vaccination resulted in lower challenge-induced IL-1α, IL-1β, and TNF-α expression than adjuvant treatment.

Th1/Th17 cytokines. In adjuvant-treated mice no Tlr4-dependent effects on post-challenge Th1/Th17 cytokines were seen (Figure 5B). In contrast, *Tlr4*-deficient wP-vaccinated mice showed lower IFN-γ, IL-17, and IL-23 expression than similarly treated wild-type animals at day 3 after challenge. In both strains of mice wP vaccination resulted in lower challenge-induced IFN-γ and IL-23 expression than adjuvant treatment, while aP vaccination resulted in lower challenge-induced expression of IFN-γ, IL-17, and IL-23 expression than adjuvant treatment.

Th2 cytokines. In adjuvant-treated mice no Tlr4-dependent effects on post-challenge Th2 cytokines were observed (Figure 5C). *Tlr4*-deficient aP-vaccinated mice showed higher challenge-induced IL-5 expression than similarly treated wild-type animals. This was also seen for wP-vaccinated animals at day 3 after challenge. In both strains of mice wP and aP vaccination resulted in higher challenge-induced IL-4 and IL-5 expression than adjuvant-treatment.

In summary, in both strains of mice wP and aP vaccination resulted in lower challenge-induced IL-1α, IL-1β, TNF-α, IFN-γ, IL-17, and IL-23 expression, and higher challenge-induced IL-4 and IL-5 expression. The exceptions to this latter picture were higher TNF-α and IL-17 expression in wP-vaccinated wild-type mice.

For *Tlr4*-deficient mice compared to wild-type controls, after challenge (i) adjuvant-treated mice showed higher expression of the pro-inflammatory cytokines IL-1α and IL-1β at day 7 after infection, (ii) wP-vaccinated mice showed lower expression of the pro-inflammatory cytokines IL-1β and TNF-α, and of Th1 and Th17 cytokines at day 3 after challenge, and (iii) wP- and aP-vaccinated mice showed higher IL-5 expression.

In short, these data show that in the lung the presence of functional Tlr4 results in a lower inflammatory response after infection, a higher inflammatory response and Th1/Th17 response after wP vaccination and challenge, and a lower Th2 response after wP and aP vaccination and challenge.

Cytokine production by bronchial lymph node cells

To investigate the role of Tlr4 in cytokine production, cell suspensions from post-challenge bronchial LN were Con A-stimulated *ex vivo*. From the panel of cytokines tested (IL-1α, IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ, and TNF-α), IL-1α and IL-13 were excluded from further analysis because for these cytokines one or more of the treatment groups showed concentrations below the detection limit for 4 or more of the 6 samples tested.

Tlr4-deficient adjuvant-treated mice showed lower IFN-γ and IL-17 production than similarly treated wild-type animals at day 7 after challenge (Figure 6). *Tlr4*-deficient wP-vaccinated mice showed lower IL-17 production than similarly treated wild-type mice at day 3 after challenge. Vaccinated wild-type mice showed lower IFN- and IL-17 production than adjuvant-treated animals at day 7 after challenge.

IL-4, IL-5, and IL-10 production was 13-, 11-, and 9-fold higher, respectively, in wP-vaccinated than in adjuvant-treated *Tlr4*-deficient mice at day 3 after challenge ($P = 0.004$, $P = 0.028$, and $P = 0.015$, respectively). No other mouse strain- or treatment-related effects on the production of these cytokines were seen. No mouse strain- or treatment-related effects on TNF- production were seen (data not shown).

Together, these data show that in the bronchial LN the presence of functional Tlr4 results in a higher Th1/Th17 response after infection, and a higher Th17 response and a lower Th2 response after wP vaccination and challenge.

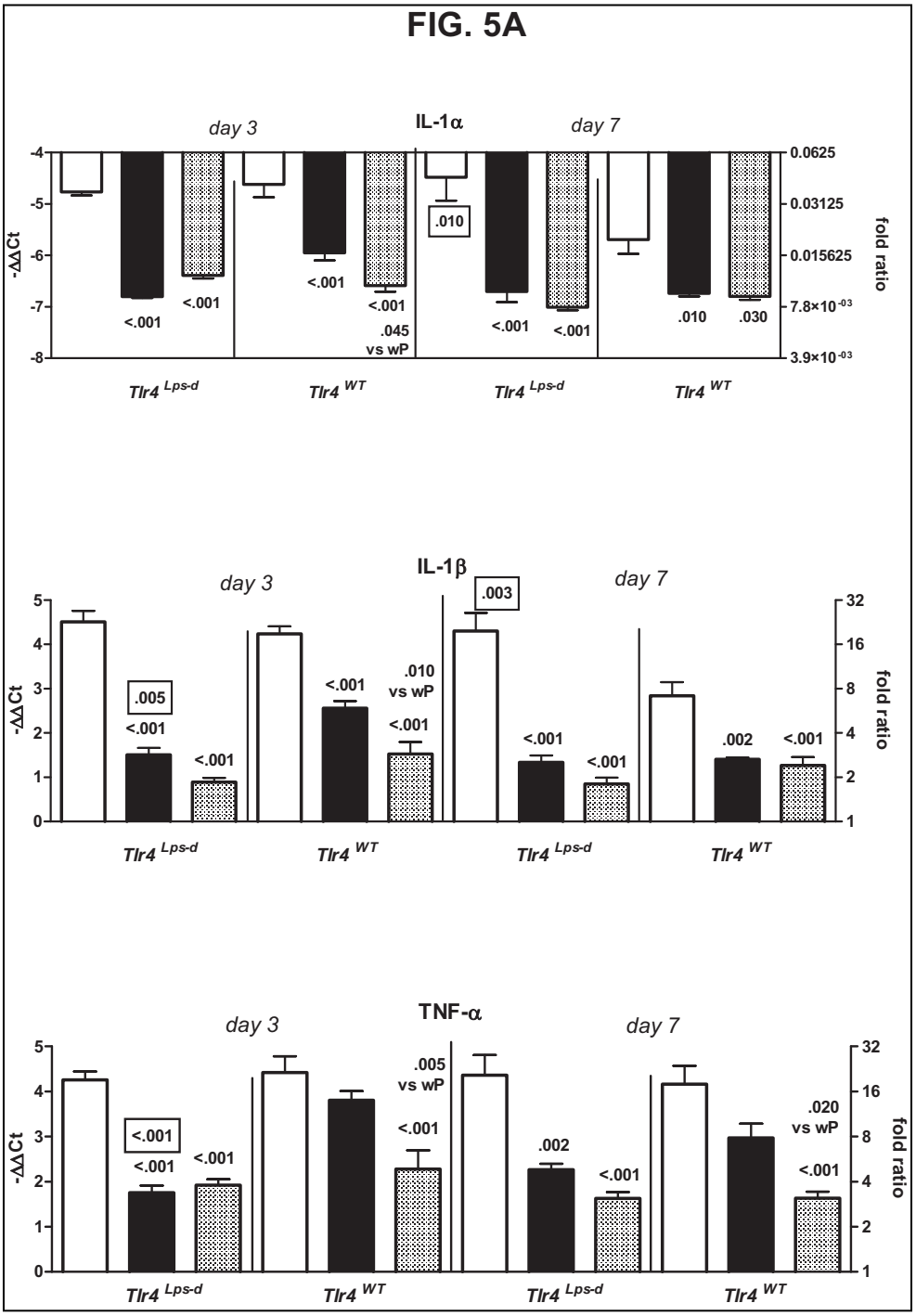
FIG. 5A
IL-1α
day 3
day 7
-ΔΔCt
fold ratio
-4
-5
-6
-7
-8
0.0625
0.03125
0.015625
7.8×10-03
3.9×10-03
<.001
<.001
<.001
<.001
.045
vs wP
.010
<.001
<.001
.010
.030
Tlr4 Lps-d
Tlr4 WT
Tlr4 Lps-d
Tlr4 WT
IL-1β
day 3
day 7
.005
<.001
<.001
<.001
.010
vs wP
<.001
.003
<.001
<.001
.002
<.001
TNF-α
day 3
day 7
<.001
<.001
<.001
.005
vs wP
<.001
.002
<.001
.020
vs wP
<.001

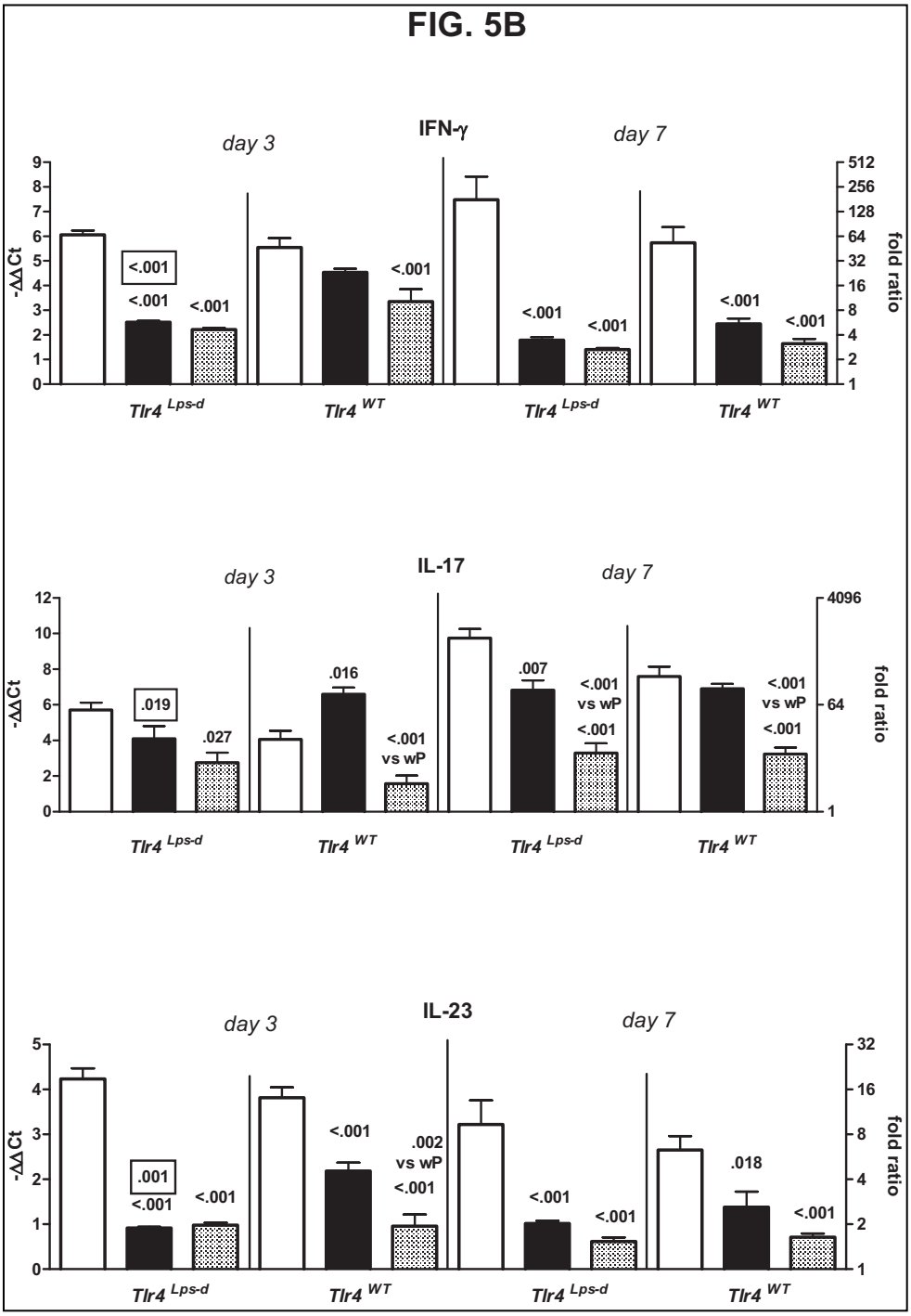
FIG. 5B
IFN-γ
day 3
day 7
-ΔΔCt
fold ratio
<.001
<.001
<.001
<.001
<.001
<.001
<.001
<.001
Tlr4 Lps-d
Tlr4 WT
Tlr4 Lps-d
Tlr4 WT
IL-17
day 3
day 7
.019
.027
.016
<.001 vs wP
.007
<.001 vs wP
<.001
<.001 vs wP
<.001
Tlr4 Lps-d
Tlr4 WT
Tlr4 Lps-d
Tlr4 WT
IL-23
day 3
day 7
.001
<.001
<.001
<.001
.002 vs wP
<.001
<.001
<.001
.018
<.001
Tlr4 Lps-d
Tlr4 WT
Tlr4 Lps-d
Tlr4 WT

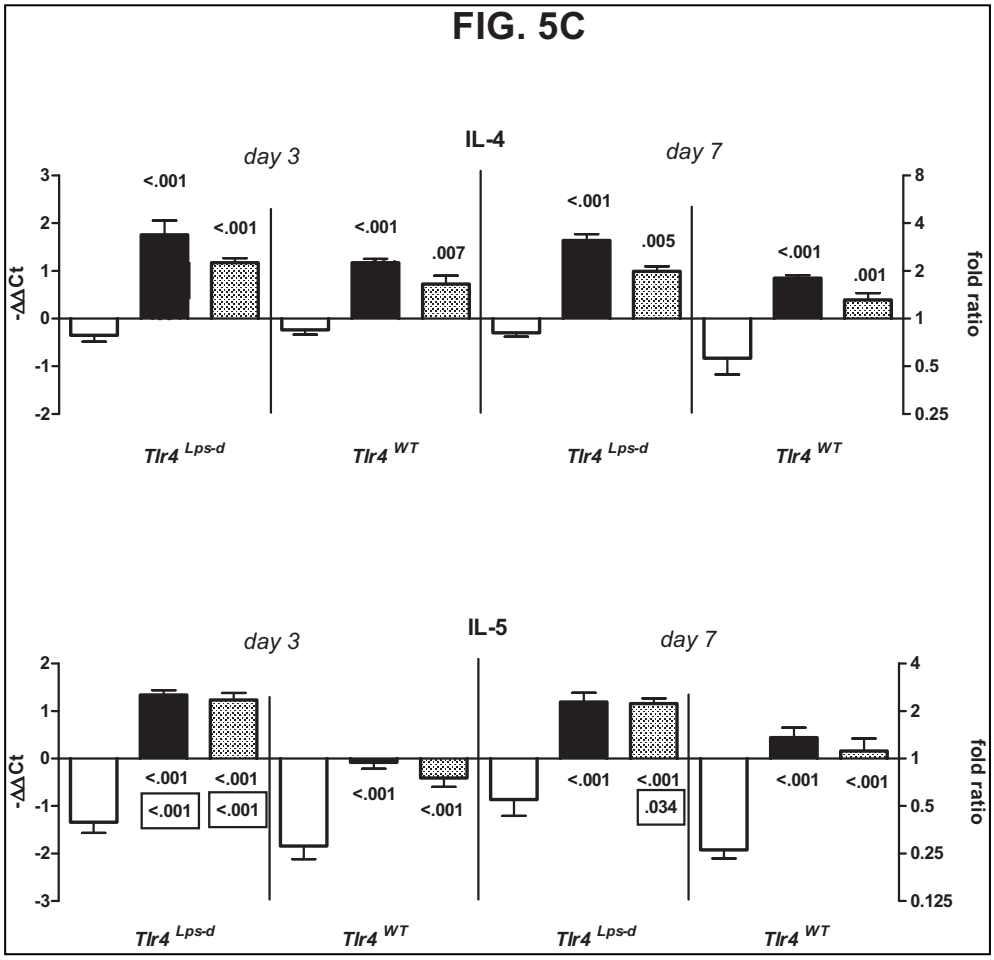

Figure 5: Cytokine expression in lung.
Tlr4-deficient and control mice were sc injected with 1/5 HD wP (|▉|), aP (|▒|), or adjuvant (| |), twice before intranasal B. pertussis infection. Three and seven days after challenge the left lung lobes were excised. RNA was extracted and analyzed by real-time PCR. The fold change in mRNA expression is relative to lung tissue of untreated C57BL/6J mice. Data are indicated as mean ± SEM (N=6). Non-boxed values show the P-value against the adjuvant control, or against the wP-vaccinated group (vs. wP). Boxed values show the P-value against similarly treated wild-type mice. ANOVA followed by Bonferroni. A single representative experiment of 2 is shown.

Cytokine production by splenocytes

Cell suspensions from post-challenge spleens were stimulated *ex vivo* with heat-killed *B. pertussis*. From the panel of cytokines tested (IL-1α, IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ, and TNF-α), IL-1α, IL-4, IL-5, IL-13, IL-17, and IFN-γ were excluded from further analysis because for these cytokines one or more of the treatment groups showed concentrations below the detection limit for 4 or more of the 6 samples tested.

Tlr4-deficient adjuvant-treated mice showed lower IL-10 and TNF-α production than similarly treated wild-type animals at both time points after challenge (Figure 6). *Tlr4*-deficient wP-vaccinated mice showed lower TNF-α production than similarly treated wild-type animals at day 7 after challenge. Vaccination failed to show effects on IL-10 production.

Together, these data show that in the spleen the presence of functional Tlr4 results in a higher inflammatory and IL-10 response after infection and a higher inflammatory response after wP vaccination and challenge.

Table 1: summary of Tlr4 mutation effects (Tlr4$^{Lps\text{-}d}$ vs. wild-type)

	Pre-challenge	**Post-challenge**	**Post-challenge**	**Post-challenge**
		Non-vaccinated	wP-vaccinated	aP-vaccinated
Number of bacteria		↑	↑	↑
Ptx-IgG	=[1]	ND[3]	ND	↓
FHA-IgG	=[2]	ND	=	=
Prn-IgG	=[2]	ND	↑	↑
Weight gain		=	=	↓
Lung pathology		=	↓	=
Pro-inflammatory cytokines[4]		↑ ↓	↓ ↓	= =
Th1/Th17 cytokines[4]		= ↓	↓ ↓	= =
Th2 cytokines[4]		= ND	↑ ND	↑ ND

[1]Only aP-vaccinated mice showed detectable Ptx-IgG levels. [2]Only wP- and aP-vaccinated mice showed detectable FHA-IgG and Prn-IgG levels. [3]ND, not detected. [4]First symbol indicates mRNA expression; second symbol indicates production.

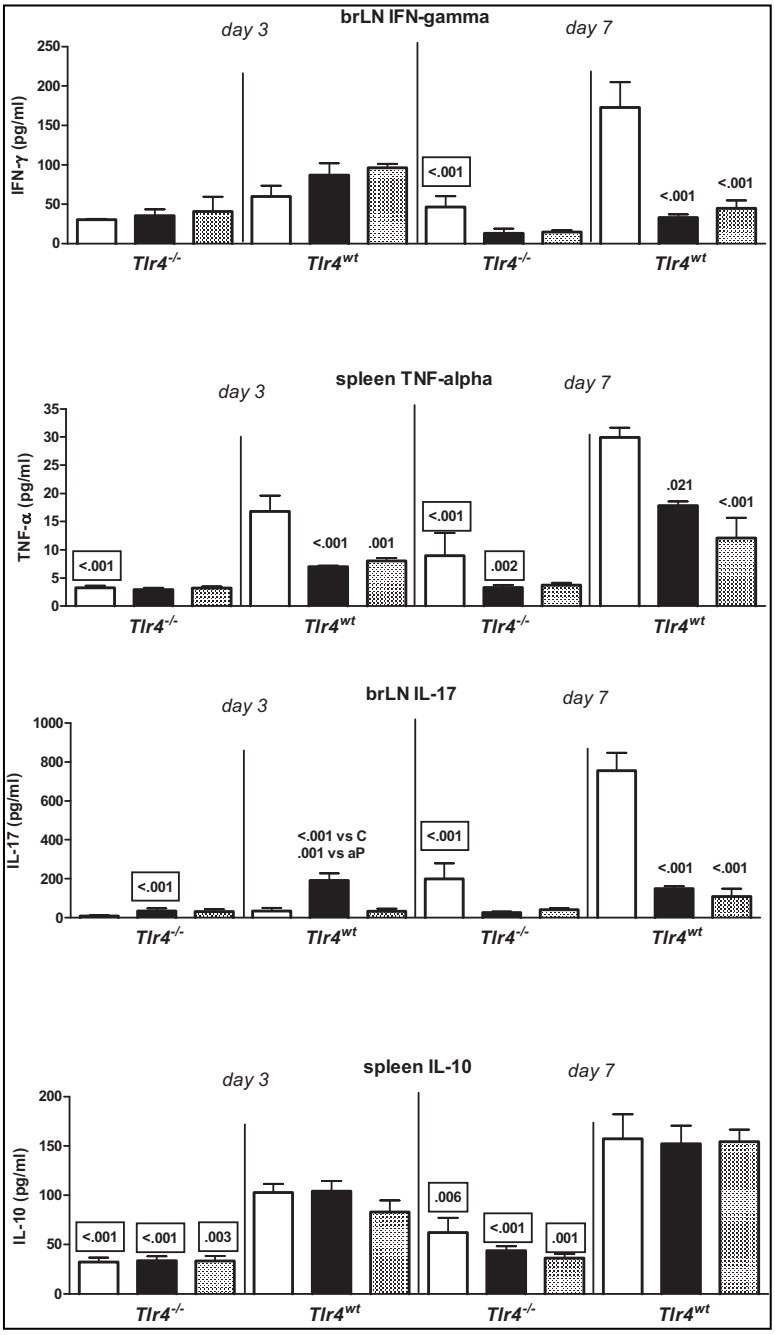

Figure 6: Ex vivo cytokine production by bronchial lymph node and spleen cells.
Tlr4-deficient and control mice were sc injected with 1/5 HD wP (|▉|), aP (|▒|), or adjuvant (| |), twice before intranasal B. pertussis infection. Three and seven days after challenge the bronchial lymph nodes (LN) and spleens were excised and cell suspensions were made. Bronchial LN cells were cultured with Con A for 24 hr; spleen cells were cultured with heat-killed B. pertussis for 72 hr. Culture supernatants were analyzed for cytokine content by Luminex. Data are indicated as mean ± SEM (N=6). Non-boxed values show the P-value against the adjuvant control, or against the wP-vaccinated group (vs. wP). Boxed values show the P-value against similarly treated wild-type mice. ANOVA followed by Bonferroni. A single representative experiment of 2 is shown.

Discussion

Here we show that in non-vaccinated *Tlr4*-deficient mice the number of bacteria is higher, expression in the lung of IL-1α and IL-1β is higher, and the production of IFN-γ and IL-17 by bronchial LN cells and TNF-α and IL-10 by splenocytes is lower than in similarly treated *Tlr4*-sufficient animals. Table 1 summarizes the findings of the present study. No effect of Tlr4 on the humoral response to vaccination (before challenge) is seen. Next, in wP-vaccinated and challenged *Tlr4*-deficient mice the number of bacteria is higher, Prn-IgG levels are higher, lung pathology is diminished, and expression in the lung of IL-1β, TNF-α, IFN-γ, IL-17, and IL-23 is lower, as is TNF-α and IL-17 production by bronchial LN cells, while IL-5 expression in the lung is higher compared to *Tlr4*-sufficient animals. In aP-vaccinated and challenged *Tlr4*-deficient mice the number of bacteria is higher, Ptx-IgG levels are lower and Prn-IgG levels are higher, weight gain is lower, and IL-5 expression in the lung is higher compared to *Tlr4*-sufficient animals. Collectively, these data show an important role for Tlr4 in events following wP vaccination, being bacterial clearance but also concomitant pathology, the induction of pro-inflammatory cytokines, and the induction of Th1, Th17, and Th2 responses. Tlr4 plays an important role also in aP vaccination, with a somewhat limited number of parameters affected, being bacterial clearance and the Th2 response. Our data confirm previous data by Higgins et al. (6), who showed an important role of Tlr4 in bacterial clearance after wP- and aP-vaccination, induction of Th1 and Th17 cells by wP but not aP vaccination, and induction of Th17 cells by infection.

On day 3 after infection vaccination resulted in a lower number of bacteria compared to adjuvant controls and this may have affected cytokine levels. Therefore, we plan to study cytokine responses earlier in infection to determine their effects on bacterial clearance.

Our data show that Tlr4 does not affect the humoral response to vaccination, and that Tlr4-dependent differences are seen only after challenge. This suggests a Tlr4-dependent effect of bacterial challenge on the humoral response, and that for this type of response Tlr4 plays a more important role in the challenge phase than in the vaccination phase. Transfer studies from vaccinated *Tlr4*-deficient mice to naive *Tlr4*-sufficient animals and challenge, or studies using conditional *Tlr4*-knockout mice should settle this issue. Our failure to detect Ptx-IgG in wP-vaccinated animals is in line with observations in both humans (9) and mice (10,11), which showed that aP vaccines induce much higher Ptx-IgG levels than wP vaccines. In contrast to a previous report where it was shown that Prn-IgG could be detected in convalescent serum from *B. parapertussis* but not *B. pertussis* infected mice (12), we did observe Prn-IgG in vaccinated mice. The lower FHA- and Prn-IgG levels after challenge may be explained by Ptx-

mediated suppression of serum antibody levels after infection (13) or by binding of the antibodies by the bacterial challenge.

Here we have shown that Tlr4 is important for the lung pathology observed after wP vaccination and challenge. Until recently, it has been assumed that tissue damage is mediated by Th1 cells (14). We have previously shown, however, that Th1 cells are not involved in lung pathology induced by wP vaccination and challenge, as T-bet KO mice that lack Th1 cells showed similar pathology compared to wild-type controls (8), suggesting that a different subset is involved. Possibly, Th17 cells may be the subset that is responsible for this pathology, as they are important in mediating tissue damage (14,15) and they are induced by wP vaccination in a Tlr4-dependent way (3). Th17 cells are induced in the presence of IL-6, while regulatory T-cells are induced in the absence of this cytokine (in an otherwise similar cytokine milieu) (15). Possibly, the induction of IL-6 by wP vaccination (7) may play a role in the generation of Th17 cells. Whether indeed Th17 cells are responsible for the observed pathology awaits further study.

We performed bronchoalveolar lavage at day 3 after infection and observed a 12-fold lower percentage of neutrophils in wP- and aP-vaccinated *Tlr4*-deficient mice than in adjuvant-treated controls ($P < 0.004$), while the percentage of lymphocytes was 17-fold higher ($P < 0.008$). In similarly treated wild-type animals we did not observe differences in the percentages of neutrophils and lymphocytes. These data suggest an impaired neutrophil response to *B. pertussis* infection in vaccinated *Tlr4*-deficient mice. Neutrophils are important in the clearance of *B. pertussis* infection in mice (16). A defective neutrophil response in vaccinated *Tlr4*-deficient mice may be an explanation for the impaired clearance in these mice.

Our finding of infection-induced IL-17 production is in line with the observation of IL-23 production by human monocyte-derived DC after *B. pertussis* infection (17). Since IL-23 is required for amplifying and/or stabilizing Th17 cells (2), both findings suggest Th17 induction by *B. pertussis* infection.

It is intriguing but also complicating that not only LPS but also Ptx may play a role in the Tlr4-dependent induction of Th17 cells. Ptx has Tlr4-dependent adjuvant activity (18,19) and is able to induce Th17 cells (20). It is likely, but not formally proven, that this latter effect is Tlr4-dependent (X. Chen, personal communication). Vaccination with wP that is derived from Ptx-deleted *B. pertussis* may clarify this issue.

In non-vaccinated controls, IFN-γ and IL-17 production by bronchial LN cells is lower when these cells are taken from infected *Tlr4*-deficient mice compared to

similarly treated wild-type animals, in line with previous observations by others for IFN-γ (4) (IL-17 was not measured in that study). These findings suggest that not only wP vaccination but also *B. pertussis* infection induces Th1 and Th17 cells in a Tlr4-dependent way.

IL-10 production by *B. pertussis*-stimulated splenocytes is lower when these cells are taken from infected *Tlr4*-deficient mice than when they are taken from similarly treated wild-type animals, in line with observations by others (4). It has been suggested that IL-10 induced by *B. pertussis* infection limits lung pathology (4). In the present study, however, we do not observe differences in challenge-induced pathology between adjuvant-treated *Tlr4*-deficient and wild-type mice. Next, while challenged-induced pathology is observed in wP vaccinated wild-type mice, and not seen in similarly treated *Tlr4*-deficient animals, IL-10 production by *B. pertussis*-stimulated splenocytes is higher in wild-type mice than *Tlr4*-deficient animals, again suggesting that in our model IL-10 is not involved in limiting pathology. It should be mentioned, however, that our observations were made earlier after challenge (day 3 compared to day 14-21).

A recent paper has shown that the number of bacteria in the lungs of *B. pertussis* infected TNF-α deficient mice was higher than in similarly treated wild-type controls (21). In the present study, *B. pertussis*-stimulated splenocytes from infected *Tlr4*-deficient mice produce less TNF-α than the same cells from similarly treated wild-type animals. The lower systemic TNF-α response observed in our study may contribute to the higher number of bacteria in the lungs of *Tlr4*-deficient mice. It has to be noted, however, that the differences in bacterial numbers between TNF-α deficient mice and their controls were not seen until day 10 after infection (21).

Two signal transduction pathways downstream of Tlr4 have been identified, the MyD88-dependent pathway and the MyD88-independent (TRIF) pathway. The former pathway involves the adaptor proteins TIRAP and MyD88, the latter one TRIF and TRAM (22). The bacterial species from which LPS originates, determines the pathway(s) activated (23). Since wP is derived from *B. pertussis*, they harbor the same LPS suggesting similar pathway activation. The wP, however, consists of glutaraldehyde-fixed bacteria, and its LPS may thus activate a different pathway than LPS from live *B. pertussis* (24). To study possible differences in pathway activation, *TRIF*-deficient (C57BL/6J-Ticam1^{Lps2}/J (25)) and wild-type controls (C57BL/6J) were compared in our model. *TRIF*-deficient mice are readily available and are not deficient in other pathways besides TRIF/TRAM, while MyD88 KO mice are not only deficient in MyD88/TIRAP but also in the IL-1R and IL-18R pathways (26). Three days after infection, wP- and aP-vaccinated mice showed a ~500-fold lower colonization than

non-vaccinated controls, and no differences in colonization were seen between TRIF-deficient and wild-type mice (data not shown). This shows that the TRIF pathway is not involved in clearance of a *B. pertussis* infection, nor is it involved in wP or aP vaccine-induced clearance.

TRIF-deficient mice are strongly impaired in LPS-induced CD40, CD80, CD86, and MHC class II upregulation, as well as type I IFN production in macrophages *in vitro*, and CD40, CD80, and CD86 upregulation in $CD11^+$ DC *in vivo*. Also, the $CD4^+$ and $CD8^+$ T-cell response is abolished when LPS is used as adjuvant (27). Importantly, type I interferons promote memory T-cell proliferation (28). Thus, the lack of an effect of *TRIF*-deficiency suggests that clearance of a *B. pertussis* infection, as well as wP- and aP-induced immunity are also functional under conditions of severely reduced costimulation and (memory) T-cell responses. This lack of an effect of *TRIF*-deficiency also suggests involvement of the MyD88-dependent pathway. We set out to study this pathway, but breeding problems with MyD88 KO mice prohibited these experiments. Still, a role for MyD88 is likely from a study by Togbe et al. (29) who showed a TIRAP- and MyD88-dependent TRIF-independent response to LPS in the lungs, including Th1 cytokine production and neutrophil influx. Both parameters are characteristic of *B. pertussis* challenge, as well as of wP but not aP vaccination and similar challenge (10,30).

Conclusion

We have shown that Tlr4 does not affect the humoral response to vaccination. Tlr4 is important in (i) the natural defense to *B. pertussis* infection, and also in the wP and aP vaccine-induced clearance, (ii) the challenge-induced lung pathology after wP vaccination, (iii) the induction of Th1 and Th17 responses, both by *B. pertussis* infection and after wP vaccination and challenge, and (iv) the reduced Th2 response after wP and aP vaccination and challenge. A summary of our findings is presented in Figure 7.

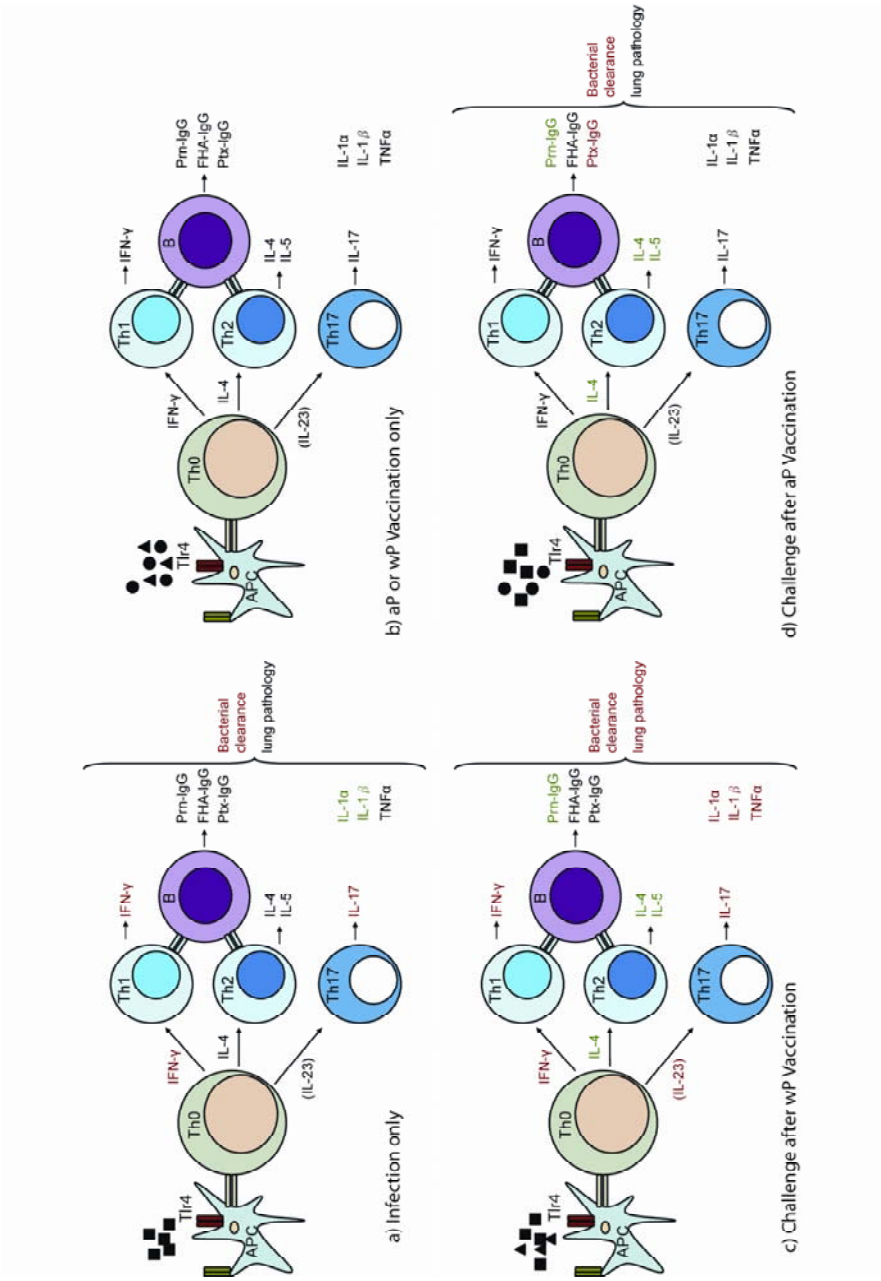

Figure 7: Effects of Tlr4 mutation in vaccine-induced immunity.
Parameters that show a lower response in Tlr4-deficient mice compared to wild-type animals are represented in red, while parameters that show a higher response are represented in green. Parameters that are not affected or have not been determined are represented in black. Triangles represent wP vaccine, circles aP vaccine, and squares B. pertussis. IL-23 is put between brackets as this cytokine is able to maintain Th17 cells, rather than generate these cells.

Authors' contributions

HAB carried out the infection and real-time PCR experiments and helped to draft the manuscript. RMS developed and carried out the serology. ERG carried out the cell culture and immunoassays. JAMAD evaluated the lung pathology. FRM participated in the study design and coordination. TGK conceived the study, participated in the study design and coordination, and helped to draft the manuscript. RJV conceived the study, participated in the study design and coordination, and wrote the manuscript. All authors read and approved the final manuscript.

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Chapter 7

A Toll-like receptor 4 polymorphism is associated with the response to whole-cell pertussis vaccination in children from the KOALA study.

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Abstract

We examined the association between haplotype-tagging SNPs in *TLR4* and the pertussis toxin-specific IgG response after whole-cell pertussis(wP) vaccination in 515 one-year-old children from the KOALA study. A lower titer was associated with the minor allele of rs2770150 supporting a role for TLR4 in the antibody response to wP vaccination.

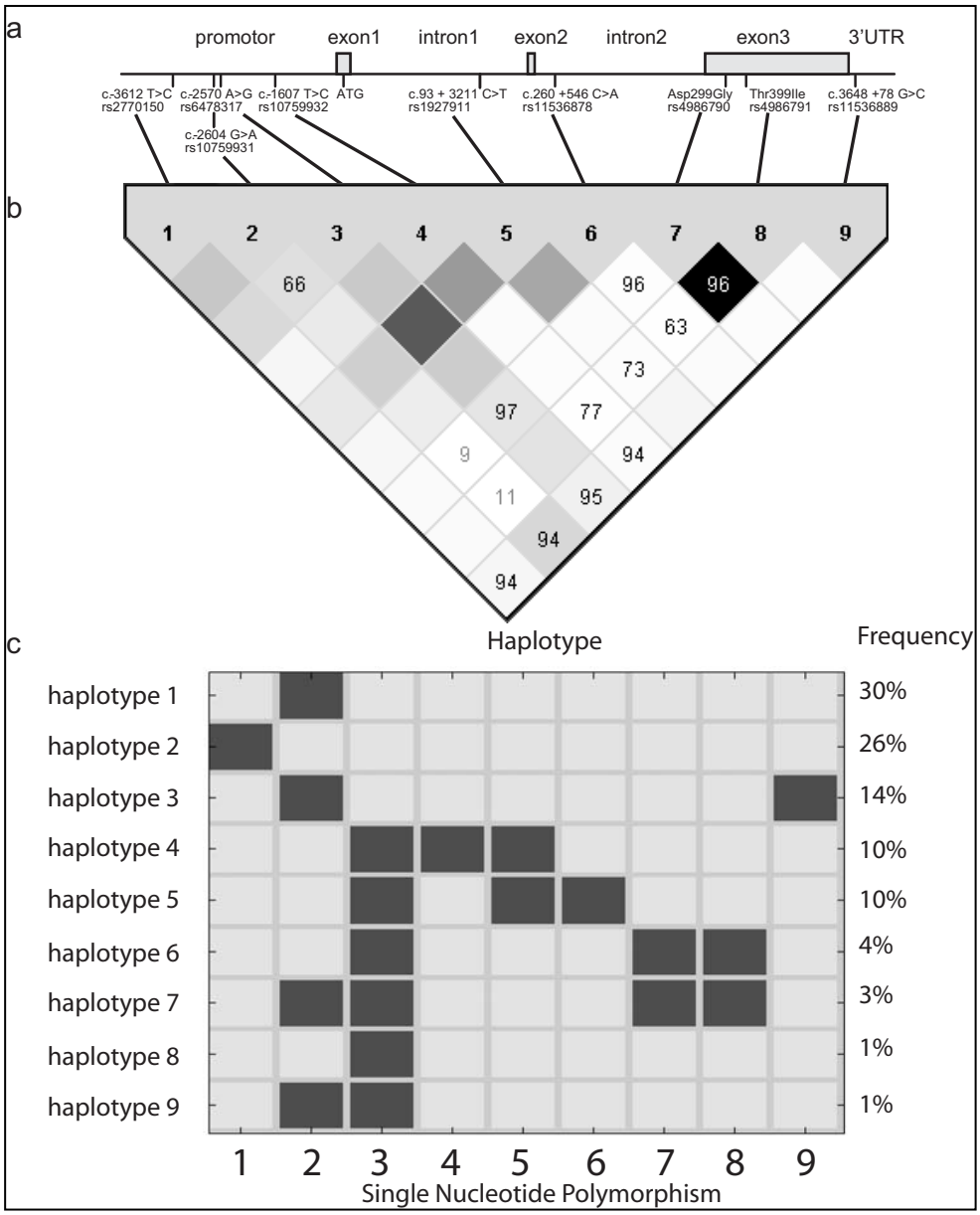

Figure 1: Position, Linkage Disequilibrium and Haplotypes of the nine SNPs within TLR4.
a) TLR4 is located on chromosome 9 (9q32) 119,506,431. Nine Haplotype-tagging SNPs were selected from the Innate Immunity web site http://www.innateimmunity.net/data/homology. All SNPs are in Hardy Weinberg equilibrium (p>0.05).
b)Pairwise Linkage Disequilibrium (LD) plot according to Haploview3.32 (7). |D'| is presented as number if it deviates from 100. |D'| is 100 when no recombination has occurred between two SNPs. The measure of r^2 is represented by color, varying from dark grey when r^2=1 (the minor alleles at two SNP positions are always present on the same haplotype), to white when r^2=0 (the minor alleles are always on separate haplotypes).
c) The frequency of the major haplotypes of TLR4 present in the KOALA cohort as identified by the nine tagging SNPs genotyped in our study. Black boxes represent minor alleles.

Body

Pertussis is a vaccine-preventable respiratory disease caused by *Bordetella pertussis.* Despite high vaccination coverage, pertussis is still endemic in most countries, including the Netherlands, with epidemic peaks that occur every 2 to 3 years (13,14,28,29). Susceptibility to *B. pertussis* and the course of infection varies between individuals. Studies in mice have provided evidence that murine host genes regulate susceptibility to *B. pertussis* infection (5,20). Animal studies furthermore indicated involvement of the toll-like receptor 4 (*Tlr4*) gene in the infection process (6,18,24). In humans, two coding variants of *TLR4* have been associated with enhanced susceptibility to infectious diseases, especially gram-negative infections, and with endotoxin hyporesponsiveness (1,2,30,33). Since *Tlr4* also plays a critical role in the response to whole-cell pertussis (wP) vaccination in mice (17) (and unpublished work (4)), we hypothesized that variation in the gene coding for TLR4 may account for some of the observed variability in the antibody response to this vaccine in humans. Furthermore, variation in response to vaccination may reflect differences in the course of infection (21). Here we studied the role of genetic variation in *TLR4* in the response to wP vaccination in the Dutch KOALA Birth Cohort Study (8,22). We therefore examined the association of SNPs in TLR4 and pertussis toxin (PT)-specific IgG following wP vaccination. The IgG antibody titer against PT, one of the dominant virulence factors of *B. pertussis,* correlates with protection against disease (12,31,32). We used vaccine-induced PT-IgG as a quantitative phenotype and compared the genotypes of high and low responders to PT. We hypothesized that minor *TLR4* alleles that may affect promoter activity or receptor affinity of TLR4 are associated with an altered IgG titer to PT.

The level of PT-IgG was determined by ELISA (15) in capillary blood collected at one year of age from 855 children. 151 children were excluded for further analysis because their parents stated in the questionnaires that the child had not received the standard pertussis vaccination (16,22). For 184 children DNA was not available, and a further five children were excluded because their PT-IgG level was above 200U/ml indicating natural infection (15). The remaining 515 children were genotyped by K-Biosciences (Cambridge, UK; http://www.kbiosciences.co.uk/) for nine SNP's located on *TLR4* (Figure 1a). After Ln-transformation the PT-IgG-levels were normally distributed according to Levene's test (P>0.05). To examine possible confounding factors we tested for associations between PT-IgG titer and the number of days between vaccination and blood sampling and infant gender using Pearson correlation. None of the tested factors influenced the PT-IgG-level (P > 0.05). All SNPs were in Hardy Weinberg equilibrium (chi-square, p>0.05). Associations between nine SNPs in *TLR4* and PT-IgG titers were assessed by ANOVA and the distribution of the genotypes among individuals with extreme 10th percentiles in PT-IgG titer was tested using Pearson's chi-square (Table 1). The rs2770150 (c.-3612 T>C SNP) was significantly associated with the lowest 10th percentile (low responders)

compared to the highest 10th percentile (high responders) titer of PT-IgG (*P*=0.027). Subjects homozygous for the minor C allele of this SNP had a significantly lower PT-IgG titer upon pertussis vaccination (*P*=0.040) compared with persons heterozygous for this allele (Figure 2).

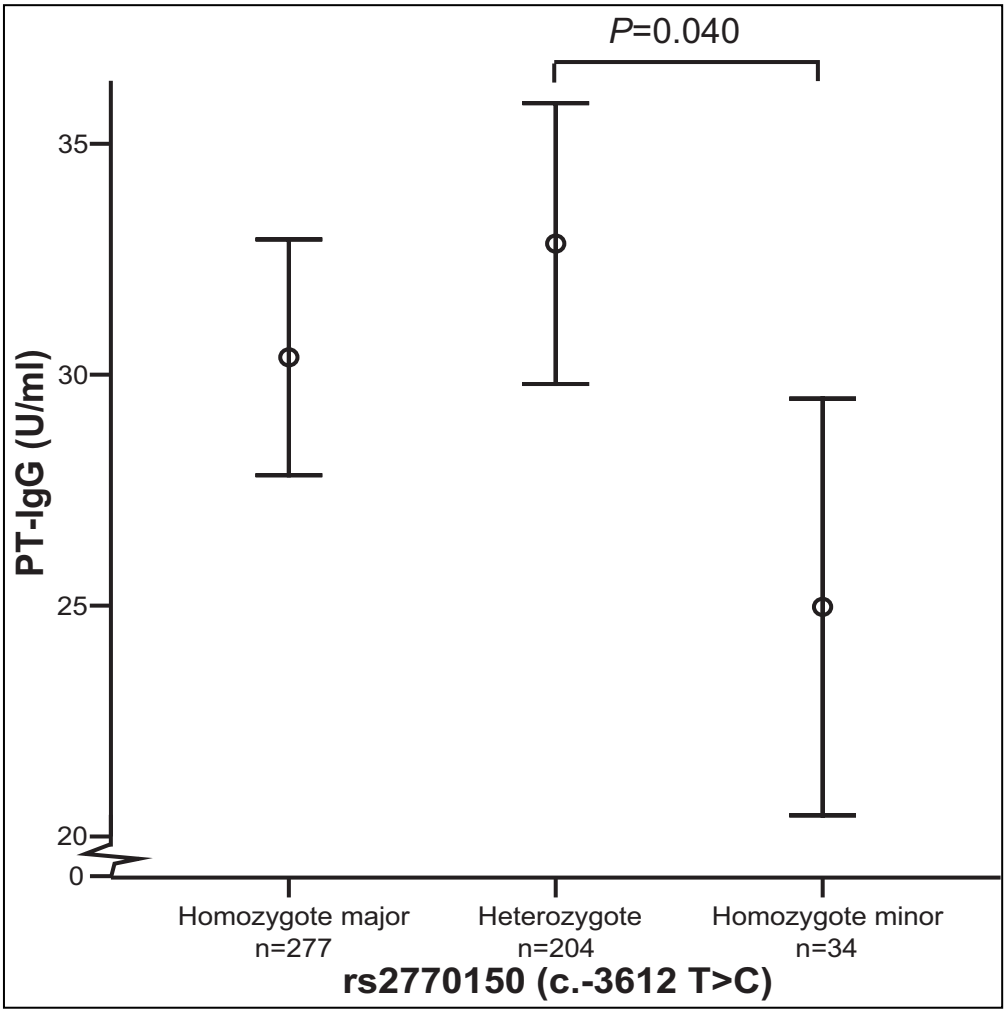

Figure 2: Pertussis toxin-specific IgG titer per genotype.
Circles represent the mean of the PT-IgG titer and the standard deviation is represented by the vertical error-bars. Horizontal lines represent a statistical difference (P<0.05) between groups according to the Least Significant Difference Post Hoc test (ANOVA, SPSS).

TABLE 1: Summary of TLR4 SNPs tested for association with PT-IgG titers.

SNP	Minor allele frequency	*P*-value		allele	*N*[1]	Ln(PT-IgG)	PT-IgG
		Continuous[2]	Percentile[3]				(U/ml)
rs2770150	0.26	0.083	**0.027**	TT	277	3.24	30.38
c.-3612 T>C [4]				CT	204	3.31	32.84
				CC	34	3.09	24.97
rs10759931	0.42	0.906	0.656	GG	187	3.26	31.03
c.-2604 G>A				GA	210	3.24	29.87
				AA	108	3.25	31.85
rs6478317	0.35	0.957	0.676	AA	203	3.26	31.40
c.-2570 A>G				GA	255	3.25	30.61
				GG	52	3.28	31.48
rs10759932	0.13	0.720	0.269	TT	365	3.25	30.71
c.-1607 T>C				CT	115	3.30	32.15
				CC	5	3.19	27.80
rs1927911	0.25	0.630	0.491	CC	276	3.25	31.25
c.93+3211 C>T				CT	207	3.23	29.49
				TT	23	3.34	33.74
rs11536878	0.12	0.620	0.649	CC	378	3.25	30.76
c.260+546 C>A				CA	103	3.24	29.80
				AA	7	3.04	21.57
rs4986790	0.07	0.836	0.548	AA	441	3.25	30.86
Asp299Gly				GA	68	3.26	30.60
				GG	3	3.45	35.33
rs4986791	0.07	0.659	0.518	CC	431	3.24	30.59
Thr399Ile				CT	66	3.30	33.29
				TT	3	3.45	35.33
rs11536889	0.14	0.308	0.502	GG	373	3.27	31.66
c.3648+78 G>C				CG	128	3.19	28.44
				CC	8	3.36	32.63

[1]Number of children per genotype.
[2]The statistical differences in phenotypes (Ln transformed PT-IgG titers) were assessed by Analysis of Variance (ANOVA).
[3]Distribution of the Pt-IgG titers among the extreme percentiles was tested by Pearson Chi-square test.
[4]SNPs were named according to the Human Genome Variation Society guidelines http://www.hgvs.org/mutnomen/recs.html.

To examine whether the effect of rs2770150 could be due to a more distant variant, we performed haplotype analysis. We constructed haplotypes for the genotyped SNPs and tested them for association with PT-IgG titers using WHAP (http://pngu.mgh.harvard.edu/purcell//whap/). There is strong linkage disequilibrium (LD) between most genotyped SNPs within *TLR4* (Figure 1b) (7). Therefore, the selected SNPs represent nine different haplotypes encompassing 99% of the haplotypes in our cohort (Figure 1c). No significant associations were found between haplotypes and the PT-IgG titer (data not shown). This could be due to the small size of our cohort, or the need for two alleles (recessive model) to obtain an effect on titer which would be missed when analyzing haplotypes.

Although all participants had PT-IgG titers that can be considered as protective at the time of blood-sampling (12) (approximately one month after receiving the fourth vaccination), *TLR4* polymorphisms may be important in the duration of protective immunity. Future work should indicate whether this and other polymorphisms in *TLR4* have clinical relevance either by affecting the antibody response following vaccination, during the waning of the antibody response, or by affecting the outcome of infection itself irrespective of vaccination.

The children in this study were vaccinated 4 times (2, 3, 4 and 11 months old) with wP vaccine that contains the TLR4 ligands LPS and PT (19,26,27). In mice *Tlr4* affected the vaccination response both after vaccination with the LPS-containing wP vaccine and the (LPS-free) acellular vaccine. Both vaccines induced less bacterial clearance in *Tlr4* defective C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$) compared to wild-type mice ($Tlr4^{Lps\text{-}n}$) (4,17), suggesting that not only the interaction between TLR4 and LPS but also the interaction between TLR4 and PT is important in the generation of vaccine-induced immunity.

The SNP that was associated with the PT-IgG titer, rs2770150, is characterized by a T to C substitution in the promoter region of TLR4 (position -3612). This SNP may therefore be involved in transcriptional regulation, suggesting that subjects with a minor allele of this SNP have a lower expression of the gene. In mice we have shown that *Tlr4*-mRNA expression is upregulated 1.5 times post *B. pertussis* infection, suggesting that transcriptional activation of *Tlr4* is involved in the response to *B. pertussis* infection (6). The results of the present study may be explained by altered transcriptional activation of TLR4 upon wP vaccination in humans.

PT-IgG levels have been shown to correlate with protection after vaccination, both in humans (12,31,32) and mice (9). Cell-mediated immunity, however, does also critically contribute to protection, both in humans (3,10,23) and mice (25). The association between PT-IgG levels and protective immunity is most apparent early after vaccination (11). We speculate that genetic diversity in *TLR4* indeed affects antibody titers after wP vaccination, but that the variation in response of one-year-old children is limited due to the many booster vaccinations. Therefore the Dutch Vaccination Program comprising four vaccinations during the first year of life may adequately answer to the genetic

variation in the most vulnerable age group, at least regarding pertussis. It remains to be established, however, whether the same holds true for the persistence of the PT-specific antibody response and the response to other vaccines.
In conclusion, we demonstrate that genetic variation in *TLR4* is associated with the wP vaccination response in one-year-old children. To our knowledge, this is the first study to report the involvement of *TLR4* in the induction of the antibody response after vaccination against *B. pertussis* in humans.

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Chapter 8

LPS analogs improve efficacy of acellular pertussis vaccine and reduce type I hypersensitivity in mice.

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Abstract
Pertussis is an infectious disease of the respiratory tract that is caused by the gram-negative bacterium *Bordetella pertussis*. Although acellular pertussis (aP) vaccines are safe, they are not fully effective and thus require improvement. In contrast to whole-cell (wP) vaccines, aP vaccines do not contain lipopolysaccharide (LPS). Monophosphoryl lipid A (MPL) and *Neisseria meningitidis* LpxL2 LPS have been shown to display immune-stimulating activity while exerting little endotoxin activity. Therefore, we evaluated whether these LPS analogs could increase the efficacy of the aP vaccine. Mice were vaccinated with Diphtheria-Tetanus-aP vaccine adjuvated with aluminum, MPL, or LpxL2 LPS before intranasal challenge with *B. pertussis*. Compared to aluminum, vaccination with either LPS analog resulted in lower colonization and a higher pertussis toxin-specific serum IgG level, indicating increased efficacy. Vaccination with either LPS analog resulted in reduced lung eosinophilia, eosinophil numbers in the bronchoalveolar lavage, and *ex vivo* production of IL-4 by bronchial lymph node cells and IL-5 by spleen cells, suggesting reduced type I hypersensitivity. Vaccination with either LPS analog increased IL-6 serum levels, although these levels remained well below the level induced by wP, suggesting that supplementation with LPS analogs may induce some reactogenicity but considerably less than wP. In conclusion, these results indicate that supplementation with LPS analogs forms a promising strategy to improve aP vaccines.

Introduction

Pertussis is caused by *Bordetella pertussis* infection of the respiratory tract and among the ten infectious diseases with the highest morbidity and mortality worldwide. After introduction of whole-cell (wP) vaccines in the 1950's, pertussis incidence has decreased significantly. Although being efficacious, wP vaccines were found to be reactogenic, leading to concerns about their safety in the 1970's. Therefore, acellular (aP) vaccines have been developed comprising purified *B. pertussis* proteins. In many countries, pertussis has re-emerged recently despite high vaccine coverage (13). Several approaches to reduce disease incidence and severity have been suggested, one of them being the improvement of existing aP.

In contrast to wP vaccines, aP vaccines are devoid of lipopolysaccharide (LPS). By engaging toll-like receptor 4 (TLR4), this molecule induces Th1 adaptive immunity (12,15,22,30,39). Consequently, concerns have been raised with respect to the relative efficacy of aP vaccines as compared with wP vaccines as well as of simultaneously administered vaccines, such as diphtheria, tetanus, polio, and *Haemophilus influenzae* b (Hib) vaccines. In fact, this concern has been substantiated by an increase in invasive Hib disease incidence in the UK that coincided with the distribution of combination vaccines that contain aP vaccine instead of wP vaccine (28). Thus, while the reactogenicity of LPS precludes its use, its adjuvanticity is regrettably missed.

We and others have shown that LPS is an essential component of wP vaccines in mice, as wP-vaccinated C3H/HeJ mice that have a point mutation in the Tlr4 gene resulting in defective signal transduction, failed to clear a *B. pertussis* challenge (21; Banus et al., manuscript in preparation). This result underlines the important role of LPS in generating a productive immune response, at least in mice. Additionally, we have shown that a functional polymorphism in TLR4 was associated with reduced pertussis toxin (Ptx)-specific IgG titers in wP-vaccinated children one year of age (Banus et al., submitted). Together, these findings strongly suggest an important role of LPS in wP vaccines.

To make use of this role of LPS, the development and use of LPS derivatives and novel LPS species have been investigated. The non-toxic LPS derivative monophosphoryl lipid A (MPL) engages TLR4 (17,33), inducing Th1 adaptive immunity and changing Th2-directed to Th1-directed responses (3,34,38,48). MPL combined with aluminum (denoted AS04) is registered for clinical use as adjuvant in viral vaccines, such as hepatitis B virus vaccine (6) and human papillomavirus vaccine (18), while MPL combined with L-tyrosine is registered for clinical use as adjuvant in allergy therapy (2,27). Furthermore, a *Neisseria meningitidis* strain deficient for the late acyltransferase LpxL2 displayed a strongly decreased endotoxic activity when tested for its capability to stimulate

human macrophages (46). This mutant LPS still exhibited immune-stimulating activity (46).

In mice, Tlr4 is critical for pertussis clearance and ensuing adaptive immunity (4,20,25). The engagement of this receptor by MPL suggests that addition of this molecule to aP vaccines may induce a vaccination response that mimics natural infection better than the aP vaccine alone, with favorable outcome. Furthermore, vaccination and particularly aP vaccination induced type I hypersensitivity, a Th2-driven response (44). Since MPL can redirect responses from Th2 to Th1, it is conceivable that this hypersensitivity may be reduced by including this molecule in the vaccine.

Here, we first investigated whether replacing aluminum by MPL in a Diphtheria-Tetanus-acellular Pertussis vaccine would beneficially affect the vaccine in a mouse model system. In a second series of experiments, also LpxL2 LPS was included as an alternative adjuvant. The clearance of a *B. pertussis* challenge, Ptx-specific serum IgG levels, parameters of type I hypersensitivity, and serum IL-6 which at elevated levels suggest reactogenicity, were determined.

Materials and Methods

Vaccines and adjuvants.

Series 1. The acellular vaccine (DTaP) was a combined vaccine consisting of diphtheria toxoid, tetanus toxoid, and a 3-component acellular pertussis vaccine (25 μg formaldehyde- and glutaraldehyde-detoxified pertussis toxin, 25 μg filamentous hemagglutinin, and 8 μg pertactin; GlaxoSmithKline, Rixensart, Belgium) in 0.5 ml saline (one human dose (HD)). The vaccine contained aluminum hydroxide as an adjuvant (< 0.625 mg aluminum/HD).

The DTaP vaccine was supplemented with two adjuvants, aluminum adjuvant (2% $Al(OH)_3$ gel (Serva, Heidelberg, Germany)) or monophosphoryl lipid A (MPL). To one HD DTaP, 2 ml aluminum/PBS (1/3 vol/vol) was added. MPL (from *Salmonella enterica* serotype minnesota Re 595) was from Sigma-Aldrich, Zwijndrecht, the Netherlands. To one HD DTaP, 2 ml of 100 μg/ml MPL/PBS was added. The amount of MPL administered is below the amount that showed no toxicological effects in a single-dose toxicity study (2).

Series 2. The same DTaP was used as for series 1.

The wP vaccine was prepared from *B. pertussis* strain B213, a streptomycin-resistant derivative of Tohama I (23). The bacteria were grown in synthetic THIJS medium (43) for 68 h at 35°C while shaking (175 rpm). The bacterial cell suspensions were heat-inactivated for 10 min at 56°C in the presence of 8 mM formaldehyde, after which the cells were collected by centrifugation for 10 min at 16,100 x *g* and resuspended in PBS to an A_{590} of 2.5, i.e., 50 international opacity units per ml (~1.6 HD/ml). The suspensions were stored at 4°C.

Before immunization, the DTaP and wP were diluted in PBS to 1/10 HD, after which 3 mg/ml aluminum phosphate (Brenntag, Dordrecht, the Netherlands), 40 μg/ml MPL (Sigma-Aldrich), or 40 μg/ml *N. meningitidis* LpxL2 LPS (46) was added as an adjuvant.

Animals. Female BALB/c mice were used at 6-8 weeks of age. They were obtained from our own breeding colony or from Harlan (Horst, the Netherlands). The diet consisted of ground standard laboratory chow (RMH-B, Hope Farms, Woerden, the Netherlands). Food and water were given ad libitum. All animal experiments were performed according to national and international guidelines.

Vaccination.

Series 1. Mice received a subcutaneous (sc) injection with 1/5 HD DTaP in 0.5 ml aluminum, 1/5 HD DTaP in 0.5 ml MPL, 0.5 ml aluminum alone, or 0.5 ml MPL alone, 28 and 14 days before infection. In one experiment, mice received a

sc injection with 1/5, 1/25, or 1/125 HD DTaP in 0.5 ml aluminum, 1/5, 1/25, or 1/125 HD DTaP in 0.5 ml MPL, 0.5 ml aluminum alone, or 0.5 ml MPL alone.
Series 2. Mice received a sc injection with 1/10 HD wP in 0.5 ml aluminum, 1/10 HD DTaP in 0.5 ml aluminum, 1/10 HD in 0.5 ml LpxL2 LPS, 1/10 HD in 0.5 ml MPL, or 0.5 ml PBS, 28 and 14 days before infection.

Bacterial strain and growth conditions. *B. pertussis* Tohama strain B213 was used. The Tohama strain has been shown to multiply in the lungs of mice (8,16,19). The bacteria were grown on Bordet Gengou (BG) agar plates supplemented with 30 μg/ml streptomycin (Tritium, Veldhoven, the Netherlands) at 35°C for 3 days. Subsequently, the bacteria were plated on BG plates without antibiotics, cultured for 3 days, resuspended in Verwey medium (NVI, Bilthoven, the Netherlands), and used for infection.

Infection of mice and autopsy. Intranasal infection was performed as described (47). Briefly, mice were lightly anaesthetized and a single drop of 40-μl inoculum containing $2x10^7$ *B. pertussis* cells was carefully placed on the top of the nose and allowed to be inhaled.

Mice were sacrificed 3, 5, or 7 days after infection. Animals were anaesthetized with ketamine, rompun, and atropine, and blood was collected from the orbital plexus. Perfusion of the right ventricle was performed with 2 ml PBS supplemented with 3.5% heat-inactivated Fetal Calf Serum (FCS; PAA, Linz, Austria). The lungs were excised and used either to obtain bronchial lymph nodes (LN) and lung lobes for enumeration of bacteria and for histological examinations, or to obtain bronchoalveolar lavage fluid (BALF) cells.

Lung lobes, CFU determination, and histological examination. A ligature was made around the right bronchus after which the right lobes were removed for enumeration of bacteria. The lobes were homogenized in 900 μl of Verwey medium using a tissue homogenizer (Pro-200, ProScientific, Monroe, CT) at maximum speed for 10 s. The homogenates were diluted in Verwey medium 10- and 100-fold for the immunized mice and 1000-fold for the control mice, and 100-μl aliquots of the dilutions were plated on BG plates supplemented with streptomycin and incubated at 35°C for 5 days. The remaining left lung lobes were fixed intratracheally using 4% formalin for 24 h. After overnight dehydration, they were embedded in paraffin. Five-μm sections were cut and stained with haematoxylin/eosin. Histological lesions were semi-quantitatively scored as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. This score incorporates the frequency as well as the severity of the lesions.

Ptx-specific IgG. Ptx-specific IgG was measured essentially as described for the analysis of human sera (14). Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were pre-coated with Ptx (NVI). Positive control serum was a

pool of sera, obtained from a previous experiment in which mice were vaccinated with DTaP plus either aluminum or MPL and challenged with *B. pertussis*. The concentration of the positive control serum was arbitrarily set at 1000 U. Dilution series of test sera and positive control sera were prepared in blocking buffer (0.5% BSA (Sigma-Aldrich, Axel, the Netherlands), 0.01% Tween-20 (Merck, Amsterdam, The Netherlands), in PBS). The plates were incubated for 2 h at 37°C and washed 3 times with 0.1% Tween-20 in PBS. The plates were then incubated with 2000-fold diluted peroxidase-labeled rabbit anti-mouse IgG (Dako, Glostrup, Denmark) in blocking buffer for 1 h at 37°C and washed. Finally, the plates were incubated with substrate solution (10% sodium acetate, 1.66% tetramethylbenzidine (Sigma-Aldrich), and 0.02% H_2O_2) for 5 min and read at 450 nm.

Total serum IgE. Blood was allowed to clot at 4°C overnight and centrifuged for 2 min at 13,000 g. Total serum IgE was measured as previously described (44).

Bronchoalveolar lavage fluid cells. A cannula was placed intratracheally and fixed using a suture. The lungs were placed in a 50-ml tube filled with PBS. One ml PBS was brought into the lung and sucked up. This was repeated twice. BALF cells were pelleted by centrifugation, resuspended in PBS, counted using a Coulter Counter (Coulter Electronics, Luton, UK), and visually differentiated after Giemsa staining.

Cell culture. The culture medium used was RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Cell suspensions were made by pressing the LN or spleens through a cell strainer (Falcon, Franklin Lakes, NJ). Cells were counted using a Coulter Counter. LN cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Concanavalin A (Con A; MP Biomedicals, Irvine, CA) in flat-bottom 12-well culture plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Spleen cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Con A or *B. pertussis* (10^5 heat-inactivated bacteria per well) in 96-well tissue culture plates (Nunc) at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Bacteria were heat-inactivated at 56°C during 30 min.

Cytokine measurements. Cytokine concentrations in the culture supernatants were measured using a 5-plex panel containing beads for mouse IL-4, IL-5, IL-10, IL-13, and IFN-γ, or an 8-plex panel containing beads for mouse IL-2, IL-4, IL-5, IL-10, IL-12p70, GM-CSF, IFN-γ, and TNF-α (Bio-Rad, Hercules, CA) as described (44). IL-6 concentrations in the sera were quantified with an ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA).

Statistics. One-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test was performed when multiple groups were compared (SPSS, Chicago, IL). The independent-samples T Test was used when two groups were compared (SPSS). Histological data were analyzed using the non-parametric Mann-Whitney U test (SPSS).

Results

***B. pertussis* colonization of the lungs.** To analyze whether supplementation of DTaP with MPL improved vaccine efficacy compared to supplementation with aluminum, mice were vaccinated twice with 1/5, 1/25, or 1/125 HD DTaP supplemented with either MPL or aluminum as adjuvant and then challenged intranasally with *B. pertussis*. Three and seven days after infection, mice were sacrificed and CFU in the lungs were enumerated. Animals treated with the adjuvants only showed a decreased number of CFU at day 7 compared to day 3 after infection (Figure 1). Vaccination with 1/5, 1/25, or 1/125 HD DTaP resulted in a reduced number of CFU at day 3 after infection, independent of the adjuvant used. Also at day 7 post infection, the vaccinated animals showed a reduced number of CFU compared to the animals treated with adjuvant only, although the difference was not significant in the case of mice vaccinated with 1/125 HD DTaP plus aluminum. Importantly, vaccination with 1/5 HD DTaP adjuvated with MPL resulted in a lower number of CFU than when the same dose DTaP adjuvated with aluminum was used, both at day 3 and day 7 after infection. At day 3 after infection this was also observed when 1/125 HD DTaP was used. In conclusion, the MPL-supplemented vaccine provided better protection than the aluminum-supplemented vaccine.

Ptx-specific IgG. Since Ptx-specific IgG titers have previously been shown to correlate with protective immunity (11,41), Ptx-specific IgG was measured in serum. When MPL was used as the adjuvant, the vaccinated animals showed a 5.4- and 2.6-fold higher Ptx-specific IgG level at days 3 and 7, respectively, compared to the mice vaccinated with DTaP plus aluminum (Figure 2). Treatment with aluminum or MPL only did not result in detectable Ptx-specific IgG levels. In conclusion, the better protection observed in the case of MPL-supplemented vaccine correlated with higher Ptx-specific IgG levels.

Evaluation of histological changes. We have previously shown that mice vaccinated with aluminum-adjuvated DTaP and subsequently challenged with *B. pertussis* revealed increased lung pathology compared to mice that were only treated with adjuvant before challenge (44). To address whether the adjuvant in the vaccine influenced lung pathology, lung histology was scored three days after infection. For both types of adjuvant, the vaccinated animals showed increased perivasculitis (P = 0.009 compared to aluminum only and P = 0.015 compared to MPL only). Eosinophilia was lower in mice vaccinated with DTaP adjuvated with MPL, compared to MPL only and compared to DTaP adjuvated with aluminum (Figure 3). Vaccination resulted in minor increases in peribronchiolitis, hypertrophy of the bronchiolar mucus cells, and alveolitis (data not shown). In conclusion, the MPL-supplemented vaccine induced lower eosinophilia than the aluminum-supplemented one, which is suggestive of a lower type I hypersensitivity.

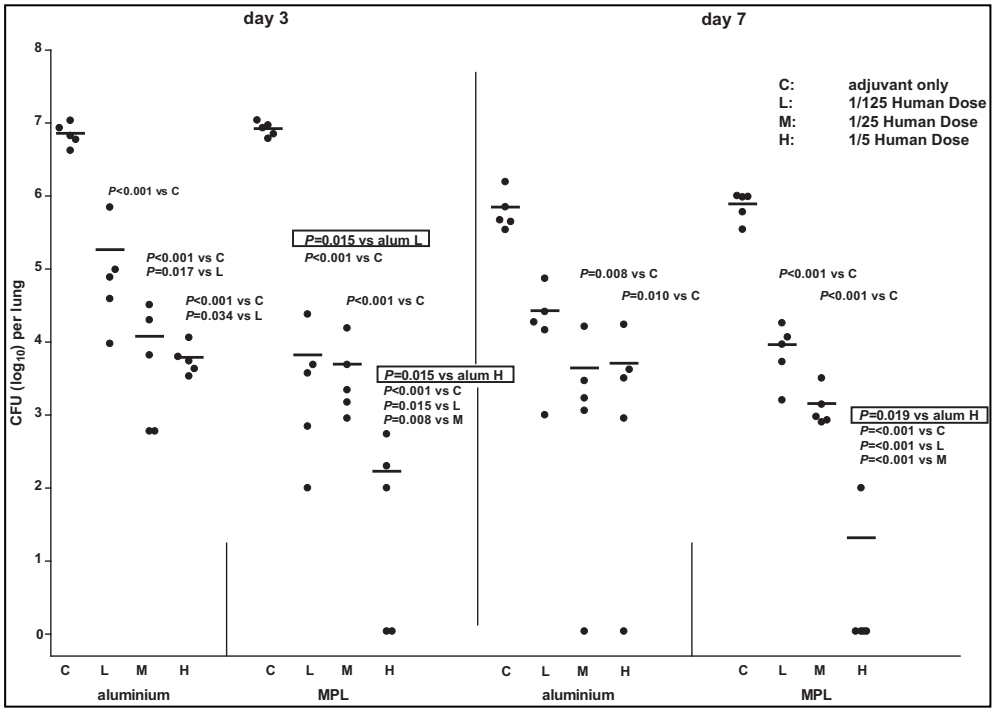

Figure 1: Colonization of the lungs by B. pertussis.
*Mice were subcutaneously injected with (**H**) 1/5, (**M**) 1/25, or (**L**) 1/125 HD DTaP plus aluminum or MPL, or (**C**) the adjuvants only, twice before intranasal B. pertussis infection. Three and seven days after infection lungs were excised, and the number of viable B. pertussis was determined in the right lung lobes. Each symbol represents the number of bacteria in the lung of an individual mouse; horizontal lines represent the group average. Non-boxed numbers show p-values when the different vaccine doses are compared, with the same adjuvant and day of sacrifice. Boxed numbers show p-values when the different adjuvants are compared, with the same vaccine dose and day of sacrifice. ANOVA followed by t-test. A single representative experiment of 3 is shown.*

Total serum IgE levels. Another parameter of type I hypersensitivity is an increased serum IgE level. We have previously shown that mice vaccinated with aluminum-adjuvated DTaP and subsequently challenged with *B. pertussis* displayed a large increase in serum IgE compared to mice that were only treated with adjuvant before challenge (44). To investigate whether supplementation of DTaP with MPL instead of aluminum resulted in lower total serum IgE, serum was taken three days after infection and analyzed. Total IgE was higher in both vaccinated groups, and no significant differences that depended on the type of adjuvant in the vaccine were seen (Figure 4). Thus, the type of adjuvant used in the vaccine did not influence total serum IgE levels.

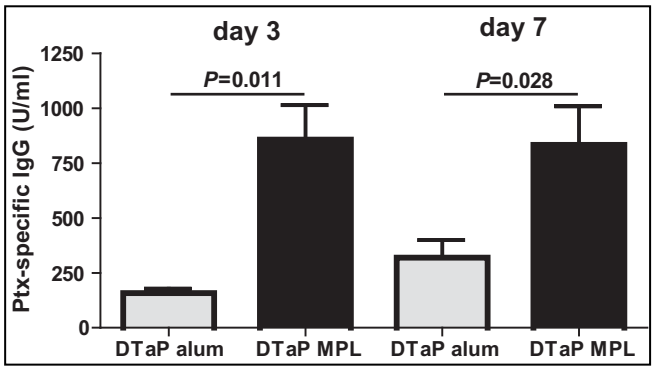

Figure 2: Ptx-specific IgG in serum.
Mice were sc injected with 1/5 HD DTaP plus aluminum or MPL, or the adjuvants only, twice before intranasal B. pertussis infection. Three and seven days after infection, mice were euthanized. Serum was taken, and serial dilutions of test and positive control sera were tested for Ptx-specific IgG. Mean ± SEM (N=5). ANOVA followed by t-test. A single representative experiment of 2 is shown.

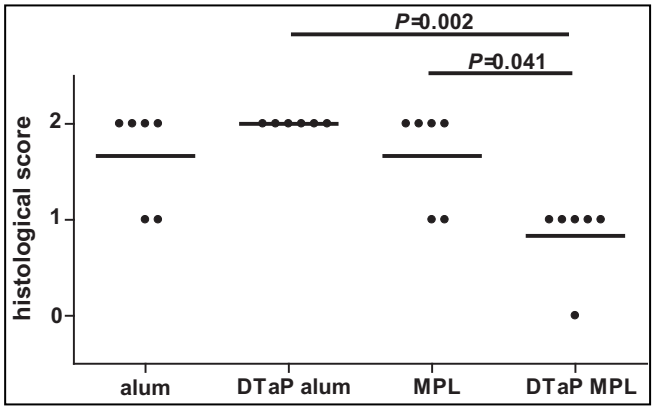

Figure 3: Lung eosinophilia.
Mice were sc injected with 1/5 HD DTaP plus aluminum or MPL, or the adjuvants only, twice before intranasal B. pertussis infection. Three days after infection, the left lung lobes were excised. Each symbol represents an individual mouse; horizontal lines represent the group average. Mann-Whitney U test. A single representative experiment of 2 is shown.

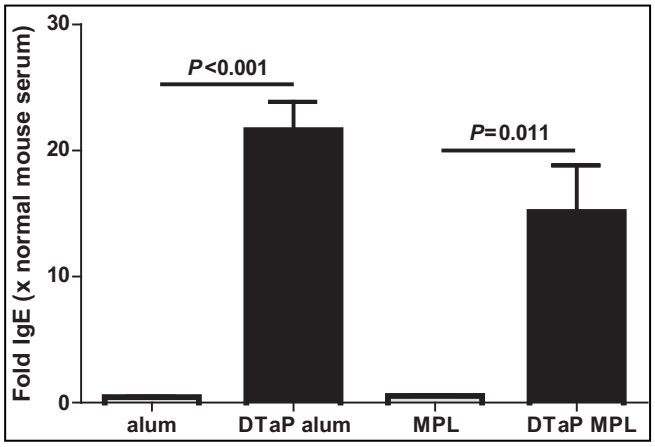

Figure 4: Total serum IgE.
Mice were sc injected with 1/5 HD DTaP plus aluminum or MPL, or the adjuvants only, twice before intranasal B. pertussis infection. Three days after infection serum was taken and assayed for total IgE. Data are indicated as mean ± SEM (N=6). ANOVA followed by Bonferroni. A single representative experiment of 2 is shown.

Cytokine production by bronchial lymph node cells. Next to lung eosinophilia, increased production of the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 was, in previous vaccination experiments with aluminum-adjuvated DTaP, suggestive of a type I hypersensitivity response (44). As MPL can redirect Th2 to Th1 responses, we reasoned that the hypersensitivity response might be lower after vaccination with the MPL-supplemented vaccine. To examine this possibility further, bronchial LN cells were cultured in the presence of Con A for 24 h and the supernatants were analyzed for cytokine content using an 8-plex assay that measured three Th2 cytokines (IL-4, IL-5, and IL-10), one Th1 cytokine (IFN-γ), and four additional cytokines involved in immune regulation (IL-2, IL-12p70, GM-CSF, and TNF-α). Bronchial LN cells from mice that were vaccinated with DTaP in aluminum showed a higher IL-4 production than those of mice that received aluminum only or of mice that were vaccinated with DTaP in MPL (Figure 5A). No treatment-related differences were seen for IL-2 and IFN-γ. The production of IL-5, IL-10, IL-12p70, GM-CSF, and TNF-α by the bronchial LN cells from mice that received aluminum only was below the detection limit. Bronchial LN cells from mice that were vaccinated with DTaP in MPL showed a higher TNF-α production than those of mice that received MPL only (P = 0.011). No further differences were seen when the production of these cytokines by bronchial LN of mice vaccinated with DTaP in MPL was compared with those of mice vaccinated with DTaP in aluminum or of mice that received MPL only (data not shown). In conclusion, vaccination with the MPL-adjuvated vaccine resulted in lower *ex vivo* Con A-induced IL-4 production by bronchial LN compared to the aluminum-supplemented vaccine, indicating that the immune response was skewed more toward a Th1-type response.

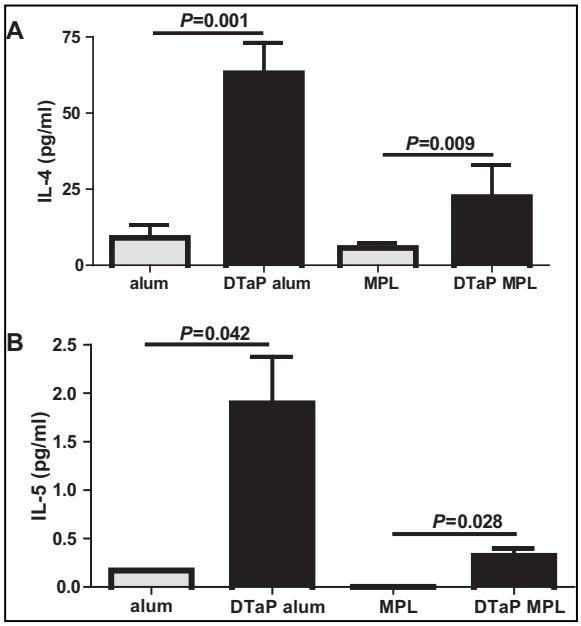

Figure 5A: IL-4 production by ex vivo Con A-stimulated bronchial LN cells. *Mice were sc injected with 1/5 HD DTaP plus aluminum or MPL, or the adjuvants only, twice before intranasal B. pertussis infection. Three days after infection, the bronchial LN were excised, the cells cultured with Con A for 24 h, and the supernatants analyzed for cytokine content.* ***5B: IL-5 production by splenocytes stimulated ex vivo with heat-killed B. pertussis.*** *The splenocytes were cultured with heat-killed B. pertussis for 72 h and the supernatants analyzed for cytokine content. Mean ± SEM (N=6). ANOVA followed by Bonferroni. A single representative experiment of 2 is shown.*

Cytokine production by splenocytes. To evaluate whether the adjuvant used in the vaccine affected *ex vivo* cytokine production by splenocytes these cells from vaccinated and control mice were isolated and cultured in the presence of heat-killed *B. pertussis* for 72 h and the supernatants were analyzed for cytokine content using a 5-plex assay that measured four Th2 cytokines (IL-4, IL-5, IL-10, and IL-13), and one Th1 cytokine (IFN-γ). Splenocytes from mice that were vaccinated with DTaP in aluminum showed a higher IL-5 production than those of mice that received aluminum only or of mice that were vaccinated with DTaP in MPL (Figure 5B). No IL-4 production was detected in splenocytes from animals that received either of the adjuvants only, whereas those of the vaccinated animals showed a mutually similar IL-4 production of ~ 7 pg/ml (data not shown). Splenocytes from all treatment groups showed a similar IFN-γ production of ~ 8 pg/ml, while IL-10 and IL-13 production could not be detected (data not shown). In conclusion, the lower *ex vivo B. pertussis*-induced IL-5 production by splenocytes from the MPL-DTaP vaccinated mice compared to the aluminum-DTaP vaccinated ones is again suggestive of a more Th1-type response.

***B. pertussis* colonization of the lungs.** The results presented so far indicate that MPL compares favorably to aluminum as an adjuvant for the DTaP vaccine. To determine whether also LpxL2 LPS would be a suitable adjuvant, a second series of experiments was performed in which mice were vaccinated twice with 1/10 HD DTaP plus either aluminum, LpxL2 LPS, or MPL before intranasal *B. pertussis* infection. Five days after infection, the mice were sacrificed and the CFU in their lungs were enumerated. When compared to the control group that received PBS, all three vaccines conferred significant protection against colonization (Figure 6). Importantly, the vaccines with LpxL2 LPS and MPL as adjuvant provided a significantly better protection than the vaccine with aluminum. Thus, with respect to efficacy, LpxL2, like MPL, compared favorably to aluminum as the adjuvant in the vaccine.

Bronchoalveolar lavage fluid cells. In order to determine whether the various adjuvants in the vaccine affected the cell-type distribution differently, lung lavage was performed five days after infection, and the BALF cells were counted and visually differentiated. The percentages and numbers of macrophages, neutrophils, and lymphocytes were similar in all groups, and also the total number of BALF cells was not differentially affected. However, the group immunized with DTaP plus aluminum showed a significantly higher number of eosinophils than all other groups (Figure 7). In conclusion, the use of LpxL2 LPS, like that of MPL, instead of aluminum as adjuvant in the DTaP vaccine resulted in a lower percentage and number of eosinophils in the BALF, which is indicative of a lower type I hypersensitivity.

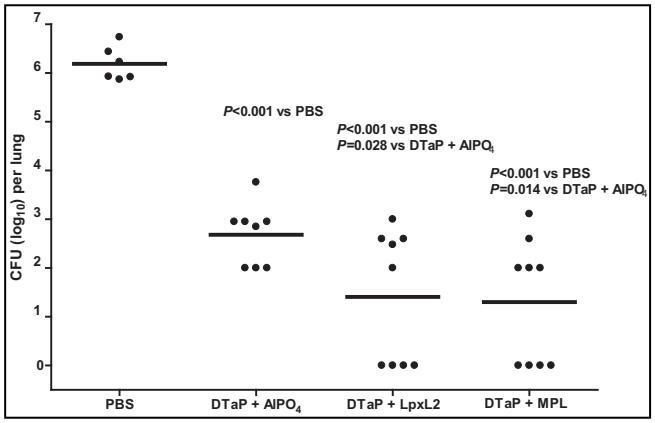

Figure 6: Colonization of the lungs by B. pertussis.
Mice were sc injected with PBS or 1/10 HD DTaP plus aluminum, LpxL2 LPS, or MPL, twice before intranasal B. pertussis infection. Five days after infection the lungs were excised, and the number of viable B. pertussis was determined. Each symbol represents the number of bacteria in the lung of an individual mouse; horizontal lines represent the group average. ANOVA followed by t-test. A single representative experiment of 2 is shown.

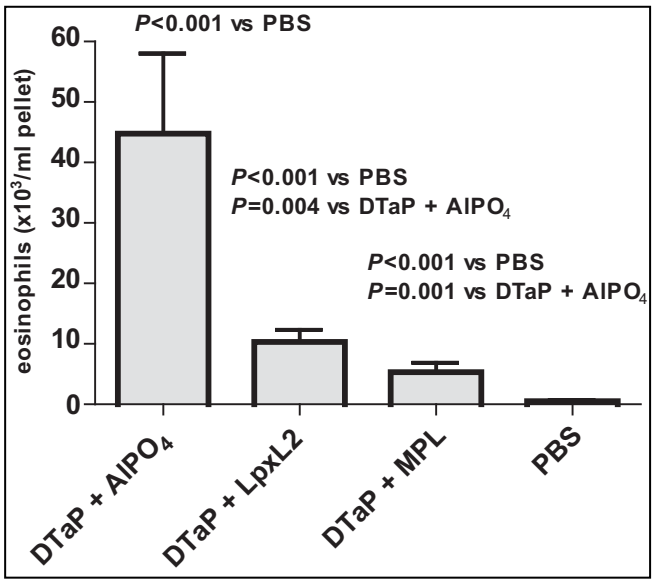

Figure 7: BALF eosinophil numbers.
Mice were sc injected with 1/10 HD DTaP plus aluminum, LpxL2 LPS, or MPL, or with PBS, twice before intranasal B. pertussis infection. Five days after infection, lung lavage was performed and the BALF cells were counted and visually differentiated. Data are indicated as mean ± SEM (N=6). ANOVA followed by Bonferroni. A single representative experiment of 2 is shown.

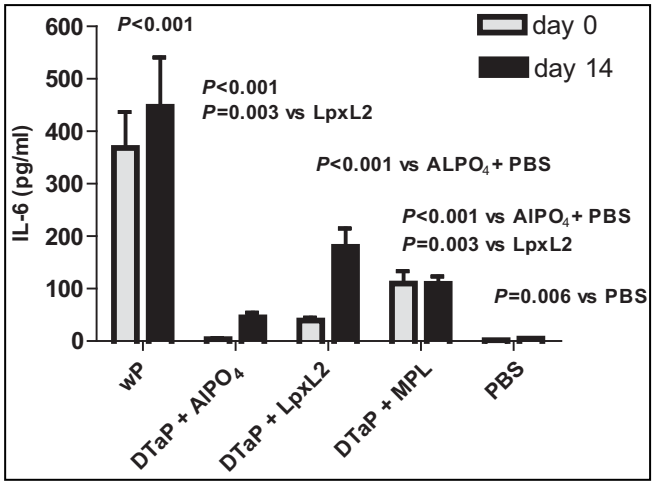

Figure 8: Serum IL-6.
Mice were sc injected with 1/10 HD wP, 1/10 HD DTaP plus aluminum, LpxL2 LPS, or MPL, or with PBS. Serum IL-6 concentrations were determined 4 h post immunization. Mean ± SEM (N=7). ANOVA followed by Bonferroni. A single representative experiment of 2 is shown.

Vaccine reactogenicity. While both LPS analogs, as compared to aluminum, improve the efficacy of the DTaP vaccine and reduce type I hypersensitivity, they should not increase its reactogenicity. To address this issue, the concentration of the pro-inflammatory cytokine IL-6 was analyzed in serum samples taken 4 h after primary or booster immunization. A group of mice, immunized with a wP vaccine, which is known to display considerable reactogenicity, was included as an additional control in these experiments. Consistent with the relatively high reactogenicity of the wP vaccine, significantly higher serum IL-6 levels were elicited in the group of mice that received this vaccine as compared to all other groups (Figure 8). The IL-6 levels elicited by aluminum-adjuvated vaccine were similar to the PBS controls. Importantly, supplementation of the vaccine with either LPS analog elicited higher IL-6 levels than with aluminum, although these IL-6 levels were considerably lower than elicited by wP. Aluminum-adjuvated vaccine elicited higher IL-6 levels during booster immunization than during primary immunization (P = 0.004); a similar effect was observed for vaccination with LpxL2 LPS-adjuvated DTaP (P = 0.007), but not the MPL-adjuvated vaccine. In conclusion, the higher IL-6 levels when using LpxL2 LPS or MPL, as adjuvant in the DTaP vaccine may suggest some reactogenicity. The considerably lower IL-6 level than the level induced by wP suggests lower reactogenicity of DTaP supplemented with LPS analogs compared to wP.

Discussion

Here, we have shown that substitution of aluminum by either one of two LPS analogs, MPL or LpxL2 LPS, as adjuvant in DTaP improves the vaccine in two ways; firstly, it enhances its efficacy, as shown by reduced colonization of the lungs after challenge and (in the case of MPL) increased Ptx-specific IgG titer; secondly, it skews the response more towards a Th1-type response as indicated by the lower Th2 cytokine production resulting in a decrease in parameters indicative of type I hypersensitivity, being lung eosinophilia and eosinophil numbers. The higher IL-6 levels induced by these supplemented DTaP vaccines compared to aluminum adjuvated DTaP may suggest some reactogenicity, although being well below the reactogenicity of wP.

We have investigated whether MPL is able to enhance vaccine efficacy by performing a dose-response analysis. While vaccination with 1/5 HD MPL-adjuvated DTaP resulted in a significantly decreased colonization compared to aluminum-adjuvated DTaP, this was not observed for 1/25 and 1/125 HD, suggesting that MPL is more effective than aluminum only at relatively high DTaP dosage. When LpxL2 LPS and MPL were compared, both LPS analogs seem to improve DTaP efficacy to a similar extent at 1/10 HD DTaP.

There is controversy regarding the correlation between Ptx-specific IgG levels and protective immunity. Ptx-specific IgG levels have been shown to correlate with protection, both in humans (11,41,42) and mice (7). Cell-mediated immunity, however, does also critically contribute to protection, both in humans (1,9,24) and mice (29). This latter notion may be an explanation of the varying association between serum levels against *B. pertussis* antigens and protective immunity. The association between Ptx-specific IgG levels and protective immunity is most apparent early after vaccination (10). We measured Ptx-specific IgG titers 17 and 21 days after the second vaccination, that is relatively early after vaccination, making it plausible that in our study indeed Ptx-specific IgG levels correlate with protection.

Using MPL as adjuvant resulted in higher Ptx-specific IgG than when aluminum was used, suggesting an improved protection. Thus, both the improved clearance of *B. pertussis* and the higher Ptx-specific IgG suggest a more efficacious vaccine when MPL is used as adjuvant in DTaP. Both in humans (5) and mice (37,45) aP vaccines induce much higher Ptx-specific IgG levels than wP vaccines. However, in the present study we compared Ptx-specific IgG levels between aP vaccinated mice only. It may be suggested that the Ptx-specific IgG levels that are induced by vaccination are affected by bacterial challenge. Pre-challenge levels are, however, similar to levels 3 and 7 days post-challenge (Stenger and Vandebriel, unpublished observations).

We have previously shown that pertussis vaccination, especially with DTaP, resulted in type I hypersensitivity. IL-4 KO mice that showed a reduced hypersensitivity response showed an unaffected clearance suggesting that the hypersensitivity is not beneficial and possibly detrimental to the host (44). As

MPL can redirect Th2 to Th1 responses (3,34,38,48), we reasoned that the hypersensitivity response might be decreased by adding MPL to DTaP. Indeed, lung eosinophilia, lung eosinophil numbers, and Th2 cytokine production were all decreased when MPL was added to DTaP. The increase in total IgE in serum was, however, unaffected by the addition of MPL. Possibly, the immune modulating capacity of MPL is too small to affect this response, or the underlying mechanism(s) of this response are (partly) different from Th2 to Th1 redirection.

LpxL2 LPS harbors strongly reduced endotoxic activity while still exhibiting some adjuvant activity compared to wild-type *N. meningitidis* LPS (46). We speculated that it might also be effective in redirecting Th2 to Th1 responses. Consistently, lung eosinophil numbers were reduced when DTaP was supplemented with LpxL2 LPS.

Levels of the pro-inflammatory cytokine IL-6 in serum samples taken 4 h after primary and booster immunization were higher when either of the LPS analogs was used as adjuvant, compared to aluminum. As expected, the IL-6 levels induced by wP were significantly higher than those induced by DTaP with any of the three adjuvants. Thus, the higher IL-6 levels induced by DTaP supplemented with either LPS may suggest some reactogenicity, albeit considerably lower than wP. Unexpectedly, aluminum-adjuvated vaccine evoked significantly higher IL-6 levels during booster immunization than during primary immunization (P = 0.004). This suggests that booster immunization, also in the absence of strong immune stimulatory molecules such as LPS, may elicit a stronger IL-6 response than primary immunization. A similar effect was observed for vaccination with LpxL2 LPS-adjuvated DTaP (P = 0.007), but not the MPL-adjuvated vaccine. This latter finding may suggest that LpxL2 LPS and MPL differ in their mechanism(s) of action.

We have chosen to measure IL-6 as parameter for reactogenicity as this cytokine was most sensitive in the response to several pyrogens in an *in vitro* system based on a human monocytic cell line and the *ex vivo* human whole blood culture test system. This latter test represents the rabbit pyrogen test (31). While low or moderate IL-6 levels form an essential part of the immune response, excessive levels may be detrimental. A level that can be taken as threshold for reactogenicity has not been established, however, and we therefore interpret increased serum IL-6 levels as being suggestive of reactogenicity.

Although MPL is believed to mimic the effects of LPS, albeit with considerably lower toxicity, differences in cytokine induction between these two molecules have been reported with MPL inducing IL-10 and IL-12, and LPS inducing only IL-12 (40). This finding may be explained by later studies, showing that MPL engages both TLR2 and TLR4, whereas LPS only acts on TLR4 (26), with TLR2 agonists inducing IL-10 and TLR4 agonists inducing IL-12 (35,36). The lack of induction of IL-1β and caspase-1 may also be an expression of the reduced toxicity of MPL compared to LPS (32). Knowledge on the mechanisms of

action of LpxL2 regarding receptor specificity and downstream effects are currently lacking.

The present study has shown that supplementation of DTaP with LPS analogs improves efficacy and reduces type I hypersensitivity. Follow-up studies are, however, required. In these studies aP adjuvated with aluminum should be compared to aP adjuvated with LPS analogs in the total absence of aluminum, a reference *B. pertussis* strain should be used for challenge, and pre- and post-challenge T-cell responses against the individual vaccine components should be measured.

In conclusion, our results demonstrate that adjuvating the DTaP vaccine with the LPS analogs MPL or LpxL2 LPS improves vaccine efficacy and redirects the immune response from a Th2- to a Th1-type response, thereby reducing type I hypersensitivity.

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Chapter 9

General discussion: Host factors in Bordetella pertussis infection and vaccination.

9.1 Introduction

"Why do individuals respond differently to infection with the same pathogen?" "Why is the response to vaccination with the same vaccine so different per person?" "Why do patients react differently to the same treatment?" Possible explanations for these differences are variable environmental factors, differences in immune status, or pathogen variation. Another likely answer to these questions is the topic of this thesis: "individuals are different" and in particular: "they carry different genetic factors". In this thesis we examine these genetic differences to learn more about pertussis.

In spite of world wide vaccination against pertussis, whooping cough is still endemic in most countries, causing 297,000 deaths annually. Whooping cough, or pertussis, is caused by an acute infection of the respiratory tract with the gram-negative bacterium *Bordetella pertussis*. Especially young non-vaccinated infants are at risk for developing severe infections with complications or even resulting in a fatal infection (89). In the Netherlands, pertussis has shown epidemic peaks every 2 to 3 years during the last decade. Since 1996 a large increase in the number of notifications, positive cultures, positive serologic results, and hospital admissions was observed (20,21). The incidence of pertussis rose suddenly from 1996 to reach 58,3 reported cases per 100 000 population in 2004, compared with 2.3/100 000 on average from 1989 to 1995 (70,71). Also in several other European countries, as well as Canada, The United States and Australia, a re-emergence of pertussis has been observed (18,19). Waning immunity, increased reporting, improved diagnosis, and adaptation of the bacterium have been proposed to explain this re-emergence. While pertussis is commonly known as a childhood disease, nowadays there has been a shift in the incidence of pertussis to adolescents and adults (5,86).

The objective of this chapter is to summarize our knowledge of genetic and genomic host factors that influence the pathogenesis of *B. pertussis* as well as the response to vaccination against pertussis. We consider that application of novel genetic and genomic techniques may identify novel insights in the pathogenesis of pertussis. There are several detailed studies which intensively describe the pathogenesis, genetic variation, virulence factors and epidemiology of pertussis (52,57,58), this chapter focuses primary on the host factors of pertussis. By increasing our insight in host factors, new methods may be developed for treatment or prevention of whooping cough. Eventually, such insights may provide novel concepts for infectious disease control by extrapolation of the knowledge acquired for *B.* pertussis to other infectious diseases.

9.2 Pathogenesis of pertussis

Biological processes of health and disease are nowadays often characterized by the involvement of biological pathways. A 'pathway' is defined as the mutual coherence of factors (such as proteins, DNA) and the sequence of communication between these factors. These biological processes are explored as complex systems of functionally interacting macromolecules. For pertussis, a substantial number of biological pathways have been identified in the past using 'classic' immunological and pathological techniques. Pathogenesis of *B. pertussis* infection is characterized by the attachment of the bacteria to ciliated epithelial cells (involving the fimbrial-like structure on the bacterial surface and cell-bound pertussis toxin), colonization of and proliferation on the ciliated mucosal cells, resulting in damage of the respiratory epithelium (by the release of toxins such as pertussis toxin which facilitates the attachment and entry of pertussis toxin into host cells (27,88)), and an acute increase in the levels of inflammatory cytokines resulting in cellular infiltrate in the alveolar spaces, bronchi and bronchioli (52,57,64). *B. pertussis* expresses various virulence factors with different functions to facilitate the infection of the host. Circulating polymorphonuclear leukocytes (PMN's) are rapidly recruited to the lungs by chemotaxis. PMN's migrate to the infection site by the detection of gradients of molecules such as interleukin-8 (IL-8), interferon gamma (IFN-gamma), and C5a. PMN's bind and ingest *B. pertussis* subsequently killing the bacteria by a combination of reactive oxygen and granule components. Finally, the PMN's undergo apoptosis, a process that appears important for the resolution of infection and inflammation (44). Toll-like receptor (TLR, a pathogen-accociated molecular pattern receptor on antigen presenting cells such as macrophages or dendritic cells) ligands such as lipopolysaccharide (LPS) are critical components for the recruitment and priming of PMN's. Serum antibody–mediated clearance of *B. pertussis* also requires a TLR-induced early recruitment of PMN's. However, pertussis toxin limits this rapid serum antibody–mediated clearance by inhibiting PMN recruitment (43).

9.3 Combining host genetics with genomics

9.3.1 Genetics

Knowledge of genetic factors that determine susceptibility or resistance to infectious diseases can be exploited to gain knowledge of infectious diseases and improve their treatment and prevention (16,40,42). Generally, there are two approaches for the identification of host genetic factors that influence the course of an infectious disease: with or without a priori. With a priori knowledge, the researcher makes a selection of the genes to be studied, based on his hypothesis that these genes could have a role in disease. Without a priori knowledge, no genes are excluded from analysis in advance. This is called a whole-genome-wide approach. In humans the identification of host genetic factors in genetic complex (or multigenic) diseases is difficult because of the small effects of individual genes and the genetic heterogeneity of the population. Most studies in humans focus on a specific pathway and study the association between single nucleotide polymorphisms (SNPs) in specific genes within such pathways and a phenotypic parameter (42). In contrast, the mouse offers significant advantages as a model to study the effects of host genetics on infectious diseases, but the path from susceptibility locus to susceptibility genes requires intensive study and many animals and the obtained results should also be extrapolated to humans (13,72).

To define numerous susceptibility genes that have small but cumulative effects on a specific phenotype, it seems that at this moment, a suitable approach is to first identify them in a mouse model, and subsequently to study the role of their human homologues in humans (24,72). To map susceptibility genes in mice, several different strategies are available encompassing different mouse models as ´genetic tool´ to facilitate linkage studies (22).

An example of such a strategy is the identification of a locus on mouse chromosome 6 that controls *Bordetella pertussis*-induced histamine sensitization (*Bphs*). This locus was identified by performing linkage analysis of congenic mice that have been administered with Ptx followed by histamine sensitization. This disease-susceptibility gene *Bphs* maps distal to the T-cell receptor beta-chain gene (81). This candidate region was subsequently reduced by testing additional markers resulting in the mapping to a cluster of genes from the TNF receptor superfamily on mouse chromosome 6 (55). Three years later it was demonstrated that this region links to the histamine H1 receptor (*Hrh1*) gene and that natural alleles of *Hrh1* control autoimmune T cell and vascular responses regulated by histamine after Ptx sensitization (48).

When our studies were initiated, the murine genome was only partially sequenced and whole genome based genetic or genomic approaches were not available at that time. We have therefore used different genetic tools and

combined this knowledge with genomic and classical immunological and pathological techniques to unravel the pathogenesis of *B. pertussis*. We started by using recombinant congenic strains (RCS) of mice (23) to study if genetic factors influence the course of pertussis and to identify possible susceptibility loci for *B. pertussis.* We described the identification of three novel susceptibility loci (9), and designated these loci *Bordetella pertussis susceptibility loci-1, -2 and -3.* For this, we screened two sets of RCS of mice for susceptibility to *B. pertussis* infection and found a wide range in bacterial numbers in the lung at 1 week post inoculation. This indicated multigenic control of the *B. pertussis* infection. We identified one locus located on chromosome 12, which we designated as *Bps-1,* and two interacting loci on chromosomes 5 and 11, designated *Bps-2 and -3*, which influence the number of bacteria in the lung 1 week after inoculation. The presence of C57BL/10 DNA in *Bps-1* instead of C3H DNA has a dominant-positive effect on the clearance of bacteria from the lung (9). We found maximum linkage between the number of CFU and the *Bps-1* locus with a LOD score of 4.6 (P = 0.000025) compared to a LOD score of 2.92 (*P=0.010)* found between the interacting loci *Bps-2* and *Bps-3* and the number of CFU's.
We have subsequently primarily focused on genes within the *Bps-1* locus because the significance of the linkage was stronger for *Bps-1* and the region was better demarcated.

9.3.2 Genomics

Omics is a general term for a broad discipline of science and engineering that analyzes the interactions of biological information objects in various omes. These include genomics, proteomics, metabolomics, transcriptomics (26).
With the introduction of whole genome expression assays (genomics or transcriptomics), 'classic' pathways have been confirmed and supplemented with mRNA expression data. It has been shown that genes that function in these pathways are upregulated during pertussis infection (7,10).
We have chosen to study the genomics of pertussis for three reasons; 1. to get more insight in the pathogenesis of pertussis on a molecular level, 2. to identify more candidate genes which play a role in the course of infection, and 3. to identify the candidate susceptibility genes within *Bps-1*.
These experiments have greatly contributed to a detailed understanding of the role of a large number of individual genes and proteins in pertussis infection. Combining these data to pathways requires advanced analysis in which bioinformatics plays a crucial role. To assign functions to differentially expressed genes and to reveal inter-gene relationships, we performed MetaCore™ analysis. MetaCore is based on a manually curated database of

human protein-protein, protein-DNA and protein-compound interactions, metabolic and signaling pathways, and the effects of bioactive molecules in gene expression. It allows the identification of affected pathways during pathologic process from microarray analysis by calculating enrichment for Gene Ontology (29) and GeneGo (56) annotated processes. We determined enrichment for these categories to identify the pathophysiological processes involved in response to *B. pertussis*. The array data are scored for pathway relevance (pathways with the highest percentage differentially expressed genes) and give specific insights in the biological processes involved (56,59).
Overall we observed that eight percent of the 22,000 genes investigated in our study was significantly differentially expressed after *B. pertussis* infection. From these genes, most upregulated genes were involved in immune-related processes, such as the acute-phase response, antigen presentation, cytokine production, inflammation, and apoptosis, while downregulated genes were mainly involved in nonimmune processes, such as development and muscle contraction. Additional pathway analysis revealed the involvement of granulocyte function, toll-like receptor signaling pathway, and apoptosis (7).

9.3.3 Combination of genetics and genomics

We have used recombinant congenic mouse strains as tool to further study the genetic basis of susceptibility to *B. pertussis* infection. The traditional approach for mapping genes in susceptibility loci is a combination of positional cloning and linkage analysis (67,75). Although this strategy has proven to be effective (22,73), the approach is quite costly and animal-consuming. Therefore, we compared the gene expression profiles in the lungs of two mouse strains, i.e. HcB-28/Dem and C3H, which showed a different course of *B. pertussis* infection, in order to further identify candidate susceptibility genes without the need for positional cloning. We hypothesized that candidate genes are differentially regulated between the two strains of mice mentioned above. By this strategy we have identified 17 genes within *Bps-1* that are differentially expressed between the two mouse strains. These genes were not regulated by *B. pertussis.* Remarkably, 17 of these genes were located in the *Bps1* region, 8 of which mapped to the *Igh* complex. Among these 8 genes were the *Igh-1* gene and genes that encode for Ig heavy chain variable regions. We observed a significantly higher expression of the gene variant of *Igh-1* isotype b in HcB mice compared to C3H mice. Because the genes that were differentially regulated between the mouse strains already showed differences in expression before infection, it appears likely that such intrinsic differences in gene regulation are involved in determining differences in susceptibility to *B. pertussis* infection.

Alternatively, such genetic differences in susceptibility may be explained by genes that are not differentially regulated between these two mouse strains or

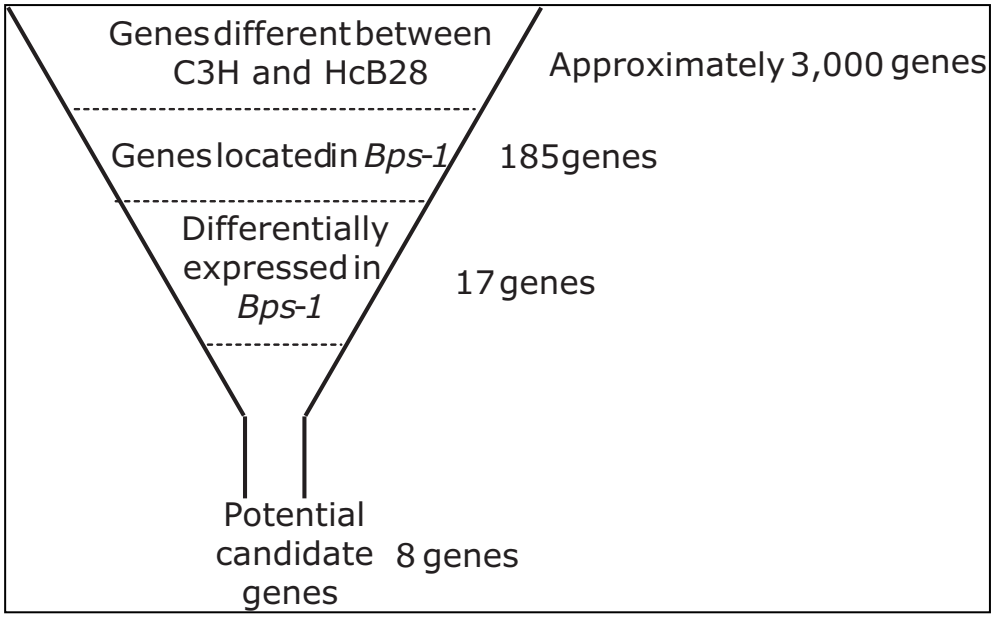

Figure 1. Approach used for identifying susceptibility genes to B. pertussis.
HcB28 mice are derived from the inbred mouse strains C3H/DISnA and C57Black/10ScSnA, resulting in 12.5% Black genome across the C3H genome. The genome of the HcB28/Dem strain, thus, differs maximally 12.5% as compared to C3H mice (Approximately 3,000 genes). Bps-1 was identified by using a F2-intercross between HcB28 and C3H mice, resulting in the description of the susceptibility locus, containing 185 genes. 17 genes in Bps-1 were differentially expressed between the two strains of mice, 8 of which mapped to the Igh complex. We propose that eight genes are potential susceptibility genes that could explain the susceptibility conferred by the Bps-1 locus.

by processes other than differential gene expression. Genes in the *Igh* complex, among which *Igh-1,* may be likely candidates to explain differences in susceptibility to *B. pertussis* as will be discussed below. By testing these RCS, we did not find *Tlr4* as major susceptibility locus while Tlr4 is repeatedly described as influencing factor in gram-negative infections (15,63). Because the RCS used were genetically identical at 33cM, chromosome 4, Tlr4 cannot be detected as susceptibility locus.

Summarizing, we have used recombinant congenic strains of mice as a tool to study the susceptibility to a *Bordetella pertussis* infection (*Figure 1*). We observed a difference in the susceptibility to *B. pertussis* between two strains of mice that are genetically different for maximally 12.5% (approximately 3000

genes). By using a F2-intercross between HcB28 and C3H mice we identified the *B. pertussis susceptibility locus-1* (*Bps-1*), containing 185 genes. In the following step, we identified 17 genes within *Bps-1* that are differentially expressed between these two strains of mice, 8 of which mapped to the *Igh* complex. Among these 8 genes were the *Igh-1* gene and genes that encode for Ig heavy chain variable regions. We can therefore state that eight genes are potential susceptibility genes that could explain the susceptibility conferring by the *Bps-1* locus. Of these eight genes *Igh-1* shows the strongest difference in gene-expression between the two strains of mice.

9.3.4 Result of the combination: Igh

As described above, we used microarray analysis of differential gene-expression between the C3H/DISnA and HcB28/Dem strains, the two strains of mice in which we identified the *Bps-1* locus, to reduce the number of candidate susceptibility genes within the *Bps1* locus (10). Genes in the *Igh* complex, among which *Igh-1*, are likely candidates to explain differences in susceptibility to *B. pertussis* (10). We observed a significantly higher expression (up to 2.8-fold) of *Igh-1* isotype b in HcB mice compared to C3H mice. The Igh locus is genetically polymorphic and very complex (78). The *Igh-1* gene exists in 2 major genetic variants (*Igh-1a* and *Igh-1b*) with 83.8% similarity (51). The *Igh-1a* allele codes for the heavy chain of IgG2a while the *Igh-1b* allele codes for the heavy chain of IgG2c (50). Mouse strains such as C57BL/6 and C57BL/10 (the donor strain of the HcB mice) only contain the *Igh-1b* variant and are therefore incapable of producing IgG2a, while mouse strains such as BALB/c only contain the *Igh-1a* variant and are therefore incapable of producing IgG2c (50,51). The oligo for *Igh-1* spotted on the microarray, was designed based on accession number XM_484178 annotated as *Igh-1a.* Because this annotation is based on the reference C57BL/6 mice, this oligo can be considered as *Igh-1b* annotated. In the sera of HcB mice we detected no IgG2a while C3H mice had significant titers of IgG2a, thereby confirming the expression results. It is tempting to speculate if and how genes within the *Igh* complex may affect differences early in the course of *B. pertussis* infection. Possible mechanisms may include differences in transcriptional gene regulation affecting immune responsiveness, different function of the IgG2a or c isotypes, or different usage of V chains. This latter possibility might imply the existence of "natural antibodies" reacting with *B. pertussis* epitopes.

It has previously been shown that genes within the *Igh-1* locus are predominantly associated with the course of a herpes simplex virus type-1 (HSV-1) infection in mice by an unknown mechanism (25,61,82). Pro-inflammatory cytokines such as IL-1β, IL-4, IL-6 and IL-7 participate in this

infection (3). Interestingly, natural killer cell activity appears to be regulated by the *Igh-1* locus but could not simply explain the differences in HSV-1 susceptibility (82). *Igh*-linked genes have further been implicated in T suppressor cell activity (47,60).
Future studies should demonstrate if *Igh-1* (and thus IgG2a / IgG2c) indeed explains the observed genetic differences between C3H and HcB mice. The functional role of Igh in the pathogenesis of *B. pertussis* can be studied in future studies such as knock-out mice or RNA interference studies.

9.4 Additional pathway information

9.4.1 Neutrophils

One of the major defense mechanisms against *B. pertussis* is the rapid recruitment of circulating polymorphonuclear leukocytes (PMN's) to the lungs to bind and ingest *B. pertussis*, subsequently killing the bacteria by a combination of reactive oxygen and granule components (52,57). As determined by molecular pathway analysis in our study, one of the most significant pathways involved in the response to *B. pertussis* infection is regulation of PMN function confirming 'classic' knowledge (7). Of the 1,841 genes that were differentially regulated upon *B. pertussis infection*, twenty genes can be ascribed to the PMN pathway, such as GM-CSF and lactoferrin The PMN pathway contains 45 genes, so, almost half of these genes are regulated upon *B. pertussis* infection (*Figure 2*). Other pathways that are upregulated by *B. pertussis* are the TLR signaling pathway (of the 47 genes involved in this pathway, 16 are *B. pertussis*-regulated), extracellular matrix remodeling (18 out of 60), and apoptosis (13 out of 36). It has been described that apoptosis of PMNs, in which TLR2 plays an important role (35), is accelerated following phagocytosis of bacteria (44,45). In conclusion, our data support a central role of PMN's in the course of *B. pertussis*.

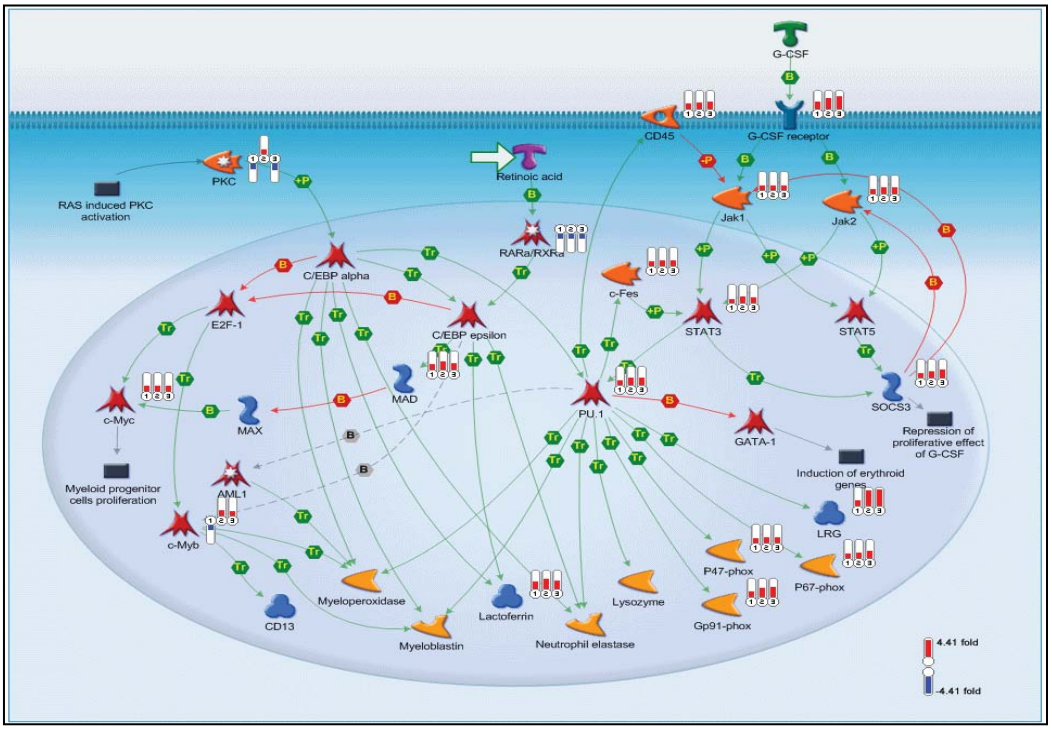

Figure 2. Regulation of polymorphonuclear granulocytes.
mRNA Expression profiles were determined from mRNA isolated from B. pertussis inoculated Mice (C3H/DISnA). Genes that were significantly different between mock- or inoculated mice (P<0.01) were included in the pathway analysis. Fold expression was defined as the difference between the expression of B. pertussis infected mice and the expression of Mock inoculated mice. Genes in the pathway that are differently expressed are represented by the fold-expression histogram. The pathway contains 45 genes, twenty of which were regulated by B. pertussis infection. The Image was generated by MetaCore™ (GeneGo).

9.4.2 Toll-like receptor signaling

Toll-like receptor 4 (TLR4) was the first TLR to be described and was originally designated human Toll (53,54). It was shown that TLR4, the receptor for lipopolysaccharide, directly influences innate immunity to gram-negative bacteria by studies in Tlr4 defective mice (15,63). Studies in mice also indicated the involvement of the toll-like receptor 4 (*TLR4*) gene in the *B. pertussis* infection process (11,33,49). It was shown that TLR4 is not only the receptor for lipopolysaccharide (LPS), but also one of the receptors for pertussis toxin (PT) (39,63,66). LPS recognition by TLR4 on dendritic cells induces IL-12 production, which supports the development of Th1 cells (38). In mice, Tlr4 is important for an effective immune response to *B. pertussis*, clearance of bacteria from the lung, and thereby reduction of lung pathology upon infection (11,33,49). Signaling through Tlr4 is also essential for induction of adaptive immunity to *B. pertussis* in response to vaccination in mice (32) (and unpublished work (8)). Whole-cell pertussis vaccination can induce the development of Th1- and Th17-cells through TLR4, which mediates protective cellular immunity to *B. pertussis* (32). In humans, two coding variants of *TLR4* have been associated with enhanced susceptibility to infectious diseases, especially gram-negative infections and endotoxin hyporesponsiveness (1,2,76,83).

In mice we demonstrated the significance of Tlr4 in genetic susceptibility and pathobiology of pertussis (11). Although the study was designed to perform a whole-genome analysis of genetic differences between different strains of mice, we came to the conclusion that all observed phenotypic differences were dominated by Tlr4. We showed that C3H/HeJ mice that carry a functional mutation in the intracellular domain of *Tlr4* showed a delayed clearance of bacteria from the lung, a higher relative lung weight, and increased body weight loss upon infection. Nine A/J mice died within the first four days after infection, while eleven C3H/HeJ mice died after four days. Remarkably, although A/J mice cleared *B. pertussis* more efficiently, we observed no significant difference in overall mortality after *B. pertussis* infection between A/J and C3H/HeJ mice. However A/J mice died earlier than C3H/HeJ mice. Thus mortality appeared associated in time with the inflammatory response.

Splenocytes from infected C3H/HeJ mice produced almost no interleukin-1beta (IL-1beta) and tumor necrosis factor alpha (TNF-alpha) upon ex vivo restimulation with *B. pertussis* and also showed a delayed gamma interferon (IFN-gamma) production. TNF-alpha expression in the lungs 3 days after infection was not affected in C3H/HeJ mice. Thus functional Tlr4 is essential for an efficient (pro) inflammatory response and efficient clearance of bacteria from the lung. However, there may be a severe price for such an efficient

proinflammatory response, as discussed above, mortality appeared associated in time with the inflammatory response.
Concluding, we showed that Tlr4 is a major genetic factor that is sufficient to explain all detectable genetic differences in bacterial clearance between these mice. Functional Tlr4 is required for an early IL-1β, TNF-α, and IFN-γ response that may enhance bacterial clearance, and thus, despite the proinflammatory nature of these cytokines, may limit pathology. This early cytokine response was fatal for ca. 50% of the mice, but the surviving mice had efficiently cleared the bacteria from the lungs, little lung pathology and rather quickly regained their body weight. A lack of this response resulted in delayed mortality, but these mice were less efficient in clearing the bacteria from the lungs, became very ill from lung edema, and only slowly regained their body weights (11).
As described above, *B. pertussis* induces pro-inflammatory cytokines by the activation of the *Toll-like receptor signaling pathway.* In addition to this 'classic' immunological cytokine measurement, we performed microarray analysis, which gives the ability to perform pathway analysis, i.e. to examine upregulation of the Toll-like receptor signaling pathway. Pathway analysis of *B. pertussis*-infected lungs of mice revealed that 16 of the 47 genes within the Toll-like receptor signaling pathway were regulated upon B. pertussis inoculation (*Figure 3*).
In humans, two functional polymorphisms Asp299Gly and Thr399Ile have been associated with infectious diseases in humans, including myocardial infection, respiratory syncytial virus infection and septic shock (76,83). These two SNPs cause a differently amino acid change (Aspartic acid to Glycine at codon 299 and Threonine to Isoleucine at codon 399) in the extracellular domain of the receptor, and they may be responsible for altered ligand binding. The role of *TLR4* in susceptibility to *B. pertussis* infection in humans is yet unknown. We therefore studied the role of *TLR4* in the response to vaccination, which is described below.

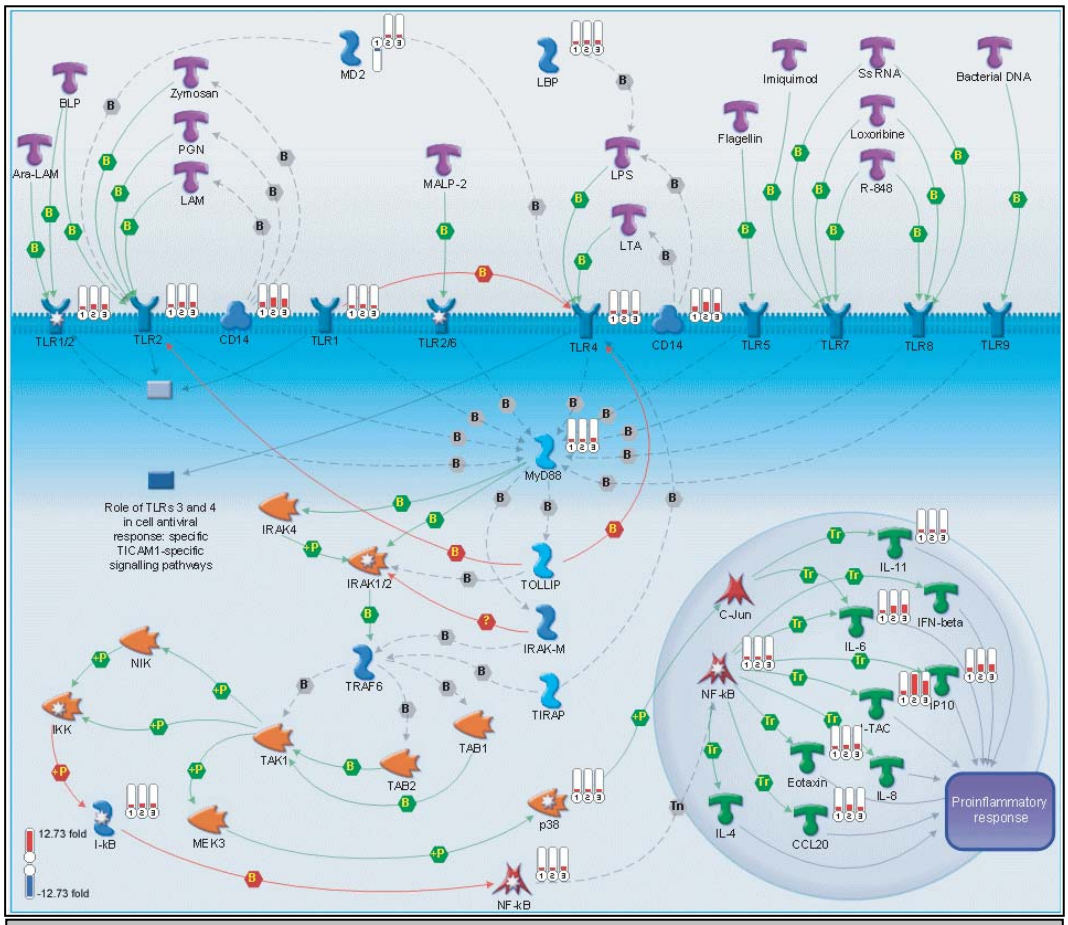

Figure 3. Toll-like receptor signaling pathway.
mRNA Expression profiles were determined from RNA isolated from the lungs of mock- and B. pertussis-inoculated mice. Genes that were significantly different between mock- and infected mice (FDR<0.05) are included in the pathway analysis. Fold expression was defined as the difference between the expression of B. pertussis infected mice and the expression of Mock inoculated mice. Genes in the pathway that are differently expressed are represented by the fold-expression histogram.The pathway contains 47 genes sixteen of which are regulated by B. pertussis. The Image was generated by MetaCore™ (GeneGo). The complete legend is presented at http://www.genego.com/files/MC_legend.pdf

9.5 Application of acquired knowledge: vaccination

9.5.1 Vaccination against pertussis in humans

Since the introduction of whole-cell pertussis vaccination in the Netherlands, mortality reduced drastically. However, the vaccine used was pyrogenic, and several side-effects were reported. Also, the effectiveness of the vaccine was not optimal and the vaccine-induced memory waned quickly. During the last decades, the incidence of pertussis increased, and the public opinion changed, and the Dutch government decided to switch from whole-cell pertussis vaccine (wP) to acellular pertussis vaccine (31,89).

It has been shown that signaling through Tlr4 is essential for induction of adaptive immunity to *B. pertussis* in response to wP vaccination in mice. Activation of innate immune cells through Tlr4 helps to induce Th1 and Th-17 cells and mediates protective cellular immunity to *B. pertussis* (8,32). Since Tlr4 plays a critical role in the wP vaccination response in mice, we considered that variation in the gene coding for TLR4 may account for some of the observed variation in the vaccine-induced antibody response in humans. We examined the influence of TLR4 on the wP vaccination response also because variation in response to vaccination may reflect differences in the course of infection (41). For this purpose a large birth cohort study (the KOALA Birth Cohort Study (46)) was studied to provide the opportunity to assess single nucleotide polymorphisms (SNPs) in *TLR4* as well as pertussis toxin (PT)-specific IgG antibodies. We showed that the c.-3612 T>C promoter SNP (rs2770150) was significantly associated with response to pertussis vaccination and that a lower antibody response was associated with the minor allele. This supports the notion that TLR4 is involved in the antibody response to wP vaccination. Future work should indicate whether this and other polymorphisms in TLR4 have clinical relevance either by affecting the antibody response following vaccination, during the waning of the antibody response, or by affecting the outcome of infection itself irrespective of vaccination. It should also be studied if TLR4 polymorphisms are also involved in the, in most countries used, acellular pertussis vaccination (aP).

9.5.2 Vaccination against pertussis in mice

To establish the mechanisms by which Tlr4 affects pertussis vaccine-induced immunity we compared Tlr4-deficient mice ($Tlr4^{LPS-d}$, C3H/HeJ) and wildtype mice ($Tlr4^{WT}$, C3H/HeOuJ) in our model of *B. pertussis* challenge after wP or aP vaccination (84).

We observed no difference in Ptx-IgG, FHA-IgG, and Prn-IgG responses after vaccination with either wP or aP. Bacterial clearance and IFN-γ and IL-17 production were lower after infection of $Tlr4^{LPS-d}$ mice compared to $Tlr4^{WT}$mice. After wP vaccination and challenge, bacterial clearance and IL-1β, TNF-α, IFN-γ, IL-17, and IL-23 expression were lower in $Tlr4^{LPS-d}$ mice compared to $Tlr4^{WT}$mice, while Prn-IgG and IL-5 expression were higher, and lung pathology was absent. After aP vaccination and challenge, IL-5 expression was higher in $Tlr4^{LPS-d}$ mice compared to $Tlr4^{WT}$ mice, while bacterial clearance and Ptx-IgG titers were lower. Thus, Tlr4 plays an important role in natural immunity, wP and aP efficacy, and the induction of Th1 and Th17 responses after wP vaccination and challenge. Tlr4 affects a more limited set of parameters in case of aP vaccination than in case of wP vaccination. The presence of functional Tlr4 is critical for lung pathology after wP vaccination and challenge, enhances pro-inflammatory cytokine production after wP vaccination and challenge, and diminishes Th2 responses after wP and aP vaccination and challenge. The humoral response to vaccination (without challenge) is not influenced by Tlr4 (Summarized in figure 4) (8). Th17 cells have recently emerged as a third independent T cell subset that may play an essential role in protection against certain extracellular pathogens (12). Th17 cells have become notorious for their involvement in a range of autoimmune diseases, but an exclusive role as mediators of pathology is unlikely to be their primary function (80). The Th17 pathway is credited for causing and sustaining tissue damage (79). The Th17-induced cytokine IL-17 stimulates the mobilization and generation of neutrophils by granulocyte-colony stimulating factor (G-CSF), thereby bridging innate and adaptive immunity. It has been suggested that this might constitute an early defense mechanism against severe trauma that would result in tissue necrosis or sepsis (80). IL-23 is important in maintaining Th17 responses but a combination of IL-6 and TGF-β acts to induce the Th17 differentiation (69).

In mice, Tlr4 affected the antibody response both after vaccination with the LPS-containing whole-cell pertussis vaccine and the (LPS-free) acellular vaccine. Both vaccines induced less protective immunity in Tlr4 defective C3H/HeJ mice ($Tlr4^{Lps-d}$) compared with the wild-type mice ($Tlr4^{Lps-n}$) (8,32), suggesting that not only the interaction between TLR4 and LPS but also the interaction between TLR4 and PT is important in the generation of vaccine-induced immunity and bacterial clearance. Our data show that Tlr4 does not affect the humoral

response to vaccination, and that Tlr4-dependent differences are seen only after challenge. This suggests a Tlr4-dependent effect of bacterial challenge on the humoral response, and that for this type of response Tlr4 plays a more important role in the challenge phase than in the vaccination phase.

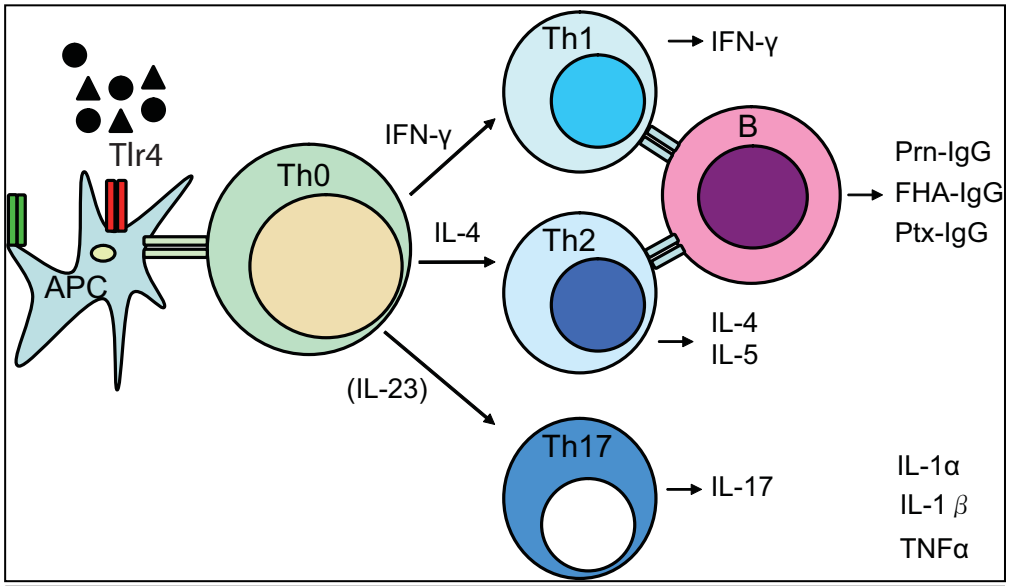

Figure 4: Schematic representation of the induction of long-term memory

9.5.3 Adjuvants

To enhance the effectiveness of vaccination, vaccines are substituted with adjuvant for the induction of long-term memory. The Dutch pertussis vaccine is adjuvated with aluminiumphosphate. The use of aluminum as adjuvant is associated with local reactions in young children (36). In the wP vaccine, a substantial amount of LPS is present, which was shown to have an adjuvant-effect on the vaccine-compontents administered simultaneously. aP vaccines lack the presence of LPS, resulting in a lower antibody response against the vaccine-components. It has been shown that LPS is an essential component of wP vaccines in mice, as wP-vaccinated C3H/HeJ mice that have a point mutation in the Tlr4 gene resulting in defective signal transduction, responded more slowly in clearing a *B. pertussis* challenge (8,32). This result underlines the important role of LPS in generating a productive immune response, at least in mice. Additionally, we have shown that a functional polymorphism in TLR4 was associated with reduced pertussis toxin (Ptx)-specific IgG titers in wP-vaccinated children one year of age (6,32). Together, these findings strongly suggest an important role of LPS in wP vaccines. LPS would therefore be an interesting adjuvant to aP vaccines, but is too toxic, and will induce unwanted side-effects such as local reactions and reactogenicity.

We have shown an application of the acquired knowledge of Tlr4 by showing that LPS analogs as adjuvant can improve acellular pertussis vaccine (28). To make use of this role of LPS, the development and use of LPS derivatives and novel LPS species have been investigated. The non-toxic LPS derivative monophosphoryl lipid A (MPL), a purified, detoxified glycolipid, engages TLR4 (62), inducing Th1 adaptive immunity and changing Th2-directed to Th1-directed responses (4,65,68). Furthermore, a *Neisseria meningitidis* strain deficient for the late acyltransferase LpxL2 displayed a strongly decreased endotoxic activity when tested for its capability to stimulate human macrophages (85). This mutant LPS still exhibited considerable immune-stimulating activity (85). Compared to aluminum only, vaccination with either LPS analog resulted in a better clearance of pertussis from the lungs, a higher pertussis toxin-specific serum IgG level, reduced lung eosinophilia, a decrease in eosinophil numbers in the bronchoalveolar lavage, and reduced ex vivo production of IL-4 by bronchial lymph node cells and IL-5 by spleen cells. Our results demonstrate that use of the acellular pertussis vaccine with the LPS analogs MPL or LpxL2 LPS as adjuvant improves vaccine efficacy and redirects the immune response from a Th2- to a Th1-type response, thereby reducing type I hypersensitivity (28).

9.6 Future prospects

Studying genetic control of susceptibility to infectious diseases may provide new tools for prevention and control of these diseases. Whole genome based genetic approaches have the major advantage that no a priori assumptions about mechanisms of pathogenesis need to be made in these studies, and thus previously unrecognized pathways of disease susceptibility can be detected (77). Disadvantages are that whole genome based genetic techniques are expensive, and powerful statistical models should be applied to avoid false positive associations (multiple testing errors).

At this moment, array-based whole-genome genotyping assays are developed covering over one million SNPs to interrogate human genetic variation (30,34,37). Such assays make whole-genome association studies more feasible and have good prospects for dissecting the genetics of common diseases, but they currently face a number of challenges, including problems with multiple testing and study design, definition of intermediate phenotypes and interaction between polymorphisms (17).

In our study, we have exploited an approach with no a priori assumptions to acquire knowledge of genetic host factors which influence the pathogenesis of *B. pertussis* and factors that influence the vaccination against pertussis.

To define the numerous susceptibility genes that have small but cumulative effects on the described phenotypes, it seems that at this moment, a suitable approach is to first identify them in a mouse model, and subsequently to study the role of their human homologues in humans (24,72). For instance, we have first identified the role of *Tlr4* in *B. pertussis* infection and vaccination in mice, and subsequently the role of *TLR4* in vaccination in children. The next step is investigation of the role of *TLR4* in human pertussis infection.

In humans (6) the identification of host genetic factors with no a priori assumptions is difficult because of the small individual effects and the genetic heterogeneity of the population. Most studies in humans focus on a specific pathway and study the association between single nucleotide polymorphisms (SNPs) of specific genes within such a pathway and a phenotypic parameter (42). In contrast, the mouse offers significant advantages as a model to study the effects of host genetics on infectious diseases with no a priori assumptions, but the path from susceptibility locus to susceptibility genes requires intensive study and many animals (13,72). One of the largest advantages of using mice as genetic tool is the possibility to reduce genetic complexity (without losing power). One of the approaches that proves the power of this principle has been described by Dr. Demant and coworkers in a series of publications. This is best illustrated for colon cancer indicating that susceptibility loci in mice may indeed

be connected to functional gene polymorphisms in humans (73-75). The study mentioned is, however, a very time- and animal-consuming method.
In our studies we have combined genetics and genomics as an approach to reduce the number of possible candidate genes to accelerate the pace and to reduce the number of animals required. This approach yielded the identification of the candidate susceptibility gene *Igh-1,* and provided more insight into the pathogenesis of pertussis.
Immunogenetic studies have provided researchers and clinical investigators crucial information that has improved our understanding of host defence against microbial pathogens. Table 1 shows examples of the effect of recent discoveries in the field of immunogenetics with foreseeable applications for the short, middle, and long term in areas such as vaccine development (14). For instance, our studies have yielded a better understanding of individual responses to vaccines by describing the role of *Tlr4* in vaccine-induced immunity after pertussis vaccination (Short term) and used this knowledge to improve the vaccine by using the innate immune adjuvants MPL and LpxL2 (Middle term).
With the development of new techniques host genetics can be studied in a broad way and with no a priori assumptions. Whole genome analysis of the human genome is no longer out of reach due to the introduction of array-based SNP assays that are able to test up to one million SNP's simultaneously. The only bottlenecks at this moment are the excessive costs, multiple testing problems and the recruitment of large cohorts. But these techniques will be more easily available in the near future (87).

	Short term	Middle term	Long term
Basic researc	Better understanding of gene function at the molecular level by the study of genetic polymorphisms	Detection of novel disease-specific genes by genome-wide scans; targeted drug discovery; gene therapy (chronic infections, inherited immune deficiencies)	
Vaccine development	Better understanding of individual responses to vaccines	Elaboration of vaccines with improved immunogenicity (use of innate immune adjuvants)	Genetic screening at birth, allowing customised vaccination programme
Preventive and predictive medicine	List of crucial polymorphisms associated with increased susceptibility to infection	Screening of individuals at high risk for infectious diseases and development of individualised prophylactic measures including antimicrobial prophylaxis; identification of new therapeutic targets	Genetic screening at birth, allowing customised prophylaxis in case of high-risk condition (immunosuppressive therapy, major surgery); development of new treatment modalities

Table 1: Examples of potential short, middle, and long-term applications and effects of innate immunogenetic studies for basic and translational research, adapted from Bochud et.al.,2007

The question remains what to do with an established association with a 'candidate gene', how to get hard evidence of the function of this gene in the pathogenesis of the disease and how to finally get rid of the prefix 'candidate'? The most common path that can lead from 'gene' to 'function' is by knocking out the candidate gene, and test whether the phenotype changes. There are several ways to knock out a gene; the traditional approach is by introducing a STOP codon in the promoter region of the gene, or by introducing an extra base pair resulting in out-of-phase transcription. The problem is that about 15 percent of these gene knockouts are developmentally lethal, meaning that the genetically altered embryos cannot grow into adult mice. This problem is often overcome through the use of conditional mutations. Conditional mutants are engineered by the introduction of a LoxP site to both flanking regions of the target gene. These LoxP sites are silent, meaning that when no action is taken, the mouse has an normal phenotype. When the mouse is, however, treated with cre recombinase, the DNA flanked by the two mutant loxP sites is deleted, causing an instant 'knock-out' of the gene.

Another method of knocking out a gene is by interference RNA (RNAi, or, post transcriptional gene silencing). RNA interference or silencing RNA is a mechanism for RNA-guided regulation of gene expression in which double-stranded RNA inhibits the expression of genes with complementary nucleotide sequences. There are several companies which provide complete assays of RNAi with targets that cover almost the complete murine genome (RNAi profiling).

When the function of a gene has been established in mice, the next logical step is to extrapolate these data to humans. Although the genomes of both, mice and men, are nowadays fully unraveled and on-line available and most murine genes can be linked to its human homologue, the evidence of the functionality of a certain gene in humans is still a difficult and time-consuming job. In addition, the roles of several genes differ between mice and humans.

For pertussis, great efforts have been made in the unraveling of the host response to infection and vaccination, especially in mice. With the introduction of the whole human genome SNP analysis, it would seem the most favorable step to study multiple vaccine-induced parameters in a large cohort of children and correlate these parameters to their whole genome SNP-profile. This strategy will presumably identify a large number of genes that are directly associated with the response to pertussis vaccination. Combining this acquired knowledge to the functional knowledge of host response of pertussis that is known so far, will open up the way to vaccine-improvement of pertussis vaccines (and perhaps of other vaccines).

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Appendices

Nederlandse Samenvatting
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Nederlandse samenvatting

"Waarom reageren mensen zo verschillend op een infectie met dezelfde ziekteverwekker?" "Waarom reageren mensen zo verschillend op vaccinatie?" "Waarom reageren patiënten zo verschillend op een behandeling?" Mogelijke verklaringen voor deze vragen zijn natuurlijk verschillende omgevingsfactoren, verschil in weerstand of verschillen in het type ziekteverwekkers. Maar ook: "Mensen zijn verschillend" en meer toegespitst: Ze hebben verschillende genen".

Ondanks dat er wereldwijd wordt gevaccineerd tegen kinkhoest, sterven er jaarlijks bijna 300.000 kinderen aan deze ziekte, en is kinkhoest nog steeds endemisch in de meeste landen. Kinkhoest wordt veroorzaakt door de bacterie *Bordetella pertussis,* en is een acute infectie van de luchtwegen. Vooral jonge, (nog) niet gevaccineerde kinderen lopen het risico om een ernstige infectie door te maken als ze in aanraking komen met deze bacterie. In Nederland vertoont kinkhoest elke 2 à 3 jaar een verhoging in incidentie in de laatste decennia. Sinds 1996 is er in Nederland een grote toename waargenomen in het aantal kinkhoest-gevallen, met de epidemie van 2004 als hoogtepunt. Ook in andere landen als Canada en de Verenigde Staten is een toename van het aantal kinkhoest-gevallen waargenomen. Wegebbende immuniteit, verbeterde rapportage, verbeterde diagnose en aanpassingen van de bacterie zijn genoemd als mogelijke verklaringen van deze waargenomen toename. Dit proefschrift onderzoekt of genetische factoren van de gastheer een rol spelen in het verloop van de ziekte kinkhoest, in de afweer tegen kinkhoest en in de reactie op vaccinatie tegen kinkhoest.

Alle lichaamscellen van de mens bevatten hetzelfde erfelijke materiaal (DNA). Toch vervullen ze elk een andere functie, of krijgen ze een andere functie als reactie op bepaalde prikkels of stress. Door bepaalde genen op het DNA geprogrammeerd 'aan' of 'uit' te zetten krijgt een cel een specifieke functie zoals het opruimen van bacteriën. Het proces om te kijken welke genen 'aan' of 'uit' staan wordt 'genomica' of 'genomics' genoemd. We kunnen op verschillende manieren kijken of genen aan of uit staan. Vroeger keken onderzoekers naar één enkel gen, tegenwoordig worden duizenden genen bestudeerd, b.v. met de microarray techniek.

Genen kunnen in de cel worden vertaald tot eiwitten zoals antilichamen of sleutelmoleculen (zoals cytokinen) die een belangrijke rol spelen in de afweer tegen infecties, of in processen zoals ontstekingen. De studie naar de codering van het DNA zelf, dus *Welke* genen liggen *waar* op het genoom?, Wat is de

variatie in genen?, Waarin verschillen ze?, en *Wat* is de functie?, wordt 'genetica' of 'genetics' genoemd. Ons uitgangspunt is dat het bestuderen van de afweer tegen kinkhoest met nieuwe technieken zoals genetics en genomics, nieuwe inzichten kan geven in het ziekteproces. Daarbij proberen we vragen te beantwoorden als: spelen genetische verschillen een rol bij het verschillende verloop van de ziekte? Kunnen we de daarvoor verantwoordelijke genen in kaart brengen en begrijpen we hoe deze het infectieproces kunnen beïnvloeden? En Kunnen we de verkregen inzichten ook toepassen, bijvoorbeeld in vaccinonderzoek?

Deze nieuwe inzichten kunnen leiden tot een verbeterde behandeling of preventie van kinkhoest.

In hoofdstuk 2 beschrijven we de identificatie van 3 nieuwe gevoeligheidsregio's (loci). Een gevoeligheidsregio, is een stukje op het genoom dat tussen verschillende individuën in verschillende vormen voor kan komen. Individuën met de éne vorm kunnen vatbaarder zijn voor een ziekte dan individuën met een andere vorm. De regio's (loci) die we hebben geïdentificeerd, hebben we *Bordetella pertussis susceptibility loci-1, -2 and -3 (Bps-1, Bps-2 en Bps-3) genoemd.* Deze loci zijn in kaart gebracht door speciale stammen laboratorium-muizen (recombinant congene muizen) waarvan het erfelijk materiaal bekend is, en die onderling een klein beetje genetisch verschillend zijn, te infecteren met exact hetzelfde aantal kinkhoest bacteriën. Een week na deze infectie is het aantal bacteriën in de long geteld als maat voor de ernst van de ziekte. Een gevoelige muis heeft meer bacteriën in de long vergeleken met een resistente muis. Vervolgens is m.b.v. een statistisch model de relatie berekend tussen de genetische verschillen van deze muizen en het aantal bacteriën in de long. Met andere woorden, we hebben onderzocht welke genetische regio's een rol spelen bij het verloop van infectie. Uit deze berekeningen bleek dat *Bps-1, -2 en -3* significant gecorreleerd zijn met de ernst van de ziekte. Deze correlatie was het meest duidelijk voor *Bps-1.* Zo'n gevoeligheidsregio is enkele miljoenen basen groot en er zitten honderden genen in. Om te bepalen welke van deze genen nu het verantwoordelijke 'gevoeligheidsgen' is, zijn vervolgstudies nodig. Een vervolgstudie is beschreven in hoofdstuk 4.

Hoofdstuk 3 gaat over de gastheer genomics van kinkhoest. Met deze studie wilden we laten zien wat er in een muizenlong gebeurt op gen-niveau nadat deze muis geïnfecteerd is met kinkhoest. Zoals hierboven beschreven is, kan een gen 'aan' of 'uit' staan in verschillende celtypes. Dit proces van aan en uit gaan verandert continu, omdat de functie van een cel kan veranderen, en zo

kan reageren op de omgeving. Het totaal aantal genen dat 'aan' of 'uit' staat in een bepaalde situatie wordt 'expressieprofiel' genoemd. Door het expressieprofiel van een cel of weefsel te vergelijken *voor* en *na* een infectie met kinkhoest, kunnen we *die genen* analyseren welke beïnvloed (gereguleerd) worden door een kinkhoest-infectie. In dit hoofdstuk hebben we muizen met kinkhoest geïnfecteerd en hebben we het expressieprofiel in de long bepaald vóór en één, drie en vijf dagen *na* de infectie. 1.841 Genen hebben een significant veranderde expressie vertoond na een kinkhoest-infectie. Met andere woorden, bijna 2.000 genen worden beïnvloed door infectie. 1.182 Genen gingen 'aan' (up-gereguleerd) en 659 genen gingen 'uit' (down-gereguleerd). De up-gereguleerde genen waren met name immunologische genen welke betrokken zijn bij de afweer tegen de bacterie, terwijl de down-gereguleerde genen vooral betrokken zijn bij niet-immunologische processen zoals ontwikkeling van de cel of spiercontractie. Van de 1.841 genen blijken er 9 in de gevoeligheidsregio *Bps-1* te liggen,13 in *Bps-2* en 62 in *Bps-3.*

In hoofdstuk 4 wordt de in hoofdstuk 2 beschreven gevoeligheidsregio *Bps-1* beter beschreven met behulp van genomics technieken. *Bps-1* is een grote regio waar 192 verschillende genen in liggen. In dit hoofdstuk willen we inzoomen in deze grote regio, en zo 1 of meerdere kandidaat gevoeligheidsgenen identificeren. Dit doen we door het expressieprofiel van de 2 muizenstammen waarmee we *Bps-1* in kaart hebben gebracht met elkaar te vergelijken. Muizenstam 1 (C3H) heeft een andere variant van *Bps-1* dan muizenstam 2 (HcB). Door de expressieprofielen met elkaar te vergelijken (dus, welke genen binnen *Bps-1* komen anders tot expressie in C3H muizen vergeleken met HcB muizen) kunnen we de functie van deze genen bestuderen, en wellicht een of meerdere kandidaat gevoeligheidsgenen aanwijzen. We hebben in totaal 206 genen gevonden die verschillend tot expressie komen tussen de 2 muizenstammen. 17 Hiervan liggen in de *Bps-1* regio. 8 Hiervan clusteren samen op het immunoglobulin heavy chain complex (Igh). Dit Igh complex codeert voor verschillende vormen van een specifiek antilichaam IgG2a of IgG2c. Igh lijkt een voor de hand liggende kandidaat als gevoeligheidsgen binnen het *Bps-1* locus.

De afweer tegen micro-organismen zoals kinkhoest, wordt op verschillende manieren geïnitieerd. Één van de manieren is de niet-specifieke (innate) immuun respons. Activatie van immuuncellen van deze respons is een belangrijke eerste stap. Deze activatie vindt plaats door herkenning van structuren van micro-organismen (zoals bijvoorbeeld een membraanmolecuul van bacteriën zoals LPS) Herkenning van deze structuren gebeurt door

receptoren op de immuuncellen. Één van deze receptoren is de Toll-like receptor 4 (Tlr4). Tlr4 is de receptor voor lipopolysaccharide (LPS), één van de componenten van de membraanstructuur van gram-negatieve bacteriën zoals *Bordetella pertussis*. Tlr4 bevindt zich op de membraan van cellen welke *B. pertussis* kunnen detecteren. In hoofdstuk 5 laten we zien dat Tlr4 een belangrijke rol speelt in het verloop van de infectie. Door muizen te infecteren die een defect hebben in Tlr4, en deze te vergelijken met *muizen* die een intact Tlr4 hebben, hebben we de functies van Tlr4 in kaart kunnen brengen. Muizen met een defect in Tlr4 blijken de bacterie slechter te kunnen klaren uit de long, hebben een hoger longgewicht (meer vocht in de long: ernstiger longontsteking), vallen meer af en hebben een vertraagde ontstekingsreactie, vergeleken met muizen welke een intact Tlr4 hebben.

In Nederland wordt er sinds 1952 gevaccineerd tegen kinkhoest, dankzij deze vaccinatie sterven er bijna geen kinderen meer in Nederland door een kinkhoest-infectie. In de laatste decennia is er wel een stijging waargenomen in het aantal kinkhoestpatiënten. Door te onderzoeken hoe individuen reageren op vaccinatie, en waarom sommige individuen anders reageren op een vaccinatie, kan meer inzicht verkregen worden in de werking van het vaccin. Kennis die wellicht kan bijdragen aan de ontwikkeling van een beter vaccin, zodat het aantal kinkhoestpatiënten afneemt.
In hoofdstuk 6 en hoofdstuk 7 wordt beschreven dat Tlr4 niet alleen een rol speelt in het verloop van de infectie met *B. pertussis*, maar dat Tlr4 ook een belangrijke rol speelt in de respons op vaccinatie tegen kinkhoest. In hoofdstuk 6 wordt dit voor muizen aangetoond, in hoofdstuk 7 voor mensen. In hoofdstuk 6 zijn wederom muizen met elkaar vergeleken die een intacte of defecte Tlr4 hebben. Deze muizen zijn gevaccineerd met whole-cell pertussis vaccin (wP) of accelulair pertussis vaccin (aP). Whole-cell pertussis vaccin, het vaccin dat in Nederland van 1952 – 2005 aangeboden werd, bestaat uit hele gedode bacteriën. Hierdoor is de opgewekte imuunrespons gericht tegen de complete bacterie. Nadeel van dit vaccin is dat membraancomponenten zoals LPS meer bijwerkingen kunnen geven zoals koorts. Vanwege de grotere kans op bijwerkingen van het wP wordt in Nederland sinds begin 2005 het acellulair pertussis vaccin aangeboden. Dit vaccin bestaat uit 3-5 gezuiverde eiwitten die ook in de bacterie voorkomen. Hoewel de imuunrespons die wordt opgewekt tegen aP minder breed is, is de opgewekte bescherming goed, en zijn de bijwerkingen minder groot. Muizen met een defect in Tlr4 hebben over het algemeen een minder goed opgebouwde of vertraagde bescherming tegen kinkhoest. Tlr4 blijkt een belangrijke rol te hebben in het opwekken van een cellulaire immuun-respons, terwijl de antistoffen die opgewekt worden

vergelijkbaar zijn. Tlr4 speelt een belangrijke rol in de effectiviteit van de vaccinatie tegen kinkhoest.

In hoofdstuk 7 hebben we onderzocht of Tlr4 niet alleen in muizen, maar ook bij kinderen een belangrijke rol speelt. De hoogte van de antilichaamrespons na vaccinatie correleert met de mate van bescherming tegen de ziekte. De antilichaamrespons van 515 kinderen is gemeten 1 maand na de 4^{e} vaccinatie. De hoeveelheid opgewekte antistoffen zijn vergeleken met verschillende genetische varianten van TLR4. Kinderen met een z.g. minor allel (de genetische variant die het minst voorkomt) van één van de variaties in TLR4, lijken vaker een lagere antistof titer te hebben. Hoewel deze kinderen nog steeds beschermd zijn, kan het zijn dat deze kinderen een sneller antilichaam-verval hebben. In vervolgstudies zal onderzocht moeten worden wat de rol van TLR4 is langer na de vaccinatie en of TLR4 ook een rol speelt in de infectie zelf bij kinderen.
Toll-like receptor 4 speelt dus een belangrijke rol in zowel de respons na een kinkhoest infectie, als in de respons na vaccinatie tegen kinkhoest. De vraag is hoe we deze kennis kunnen gebruiken om te komen tot een verbeterde behandeling of preventie van kinkhoest. Een voorbeeld hiervan is gedemonstreerd in hoofdstuk 8. In dit hoofdstuk wordt beschreven dat het door Tlr4 gedetecteerde lipopolysaccharide (LPS) niet alleen *ongewenste* bijwerkingen heeft indien dit aanwezig is in een vaccin, maar dat het ook een versterkende werking heeft op de geïnduceerde bescherming tegen zowel de kinkhoestbacterie zelf als ook tegen andere bacteriën in het zelfde vaccin (difterie, tetanus en rode hond). In hoofdstuk 8 wordt een gedeelte van LPS (het niet toxische deel) toegevoegd aan het acelullaire pertussis vaccin. Muizen die gevaccineerd zijn tegen kinkhoest, met het toegevoegde LPS-analoog hebben minder bacteriën in de long (en zijn dus beter beschermd) en vertonen minder bijwerkingen. Het toevoegen van een LPS-analoog aan bestaande pertussis vaccins werkt dus via Tlr4, en lijkt een goede manier te zijn voor de verbetering van het pertussis vaccin.

Samengevat onderzoekt dit proefschrift de rol van de gastheer en genetische verschillen tussen gastheren in het verloop van de ziekte kinkhoest. Het laat zien dat deze factoren ook een rol kunnen spelen in de vaccinatierespons. Fundamentele genetische studies beginnen vaak in proefdiermodellen (zoals de muis) en het is niet altijd eenvoudig om de gevonden resultaten te extrapoleren naar de mens. Wij laten zien dat Tlr4 niet alleen een rol speelt in de vaccinatierespons bij muizen, maar ook bij mensen. Deze kennis kan bijdragen in de ontwikkeling van een verbeterd kinkhoest vaccin, b.v. door de toevoeging van LPS-analogen aan bestaande vaccins.

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Curriculum Vitae

The author of this thesis was born on July 20th 1978 in Utrecht, the Netherlands. After finishing high school at the Christian College Zeist in 1995 he studied Biochemistry at the department of Laboratory Science from the Faculty of Science and Engineering of the Hogeschool van Utrecht. During this study he completed his internship in the Virology group of the Research Laboratory of Infectious Diseases (LIO) at the National Institute for Public Health and the Environment (RIVM). In 1999 he graduated and he started as technician in the Bacteriology group of the Research Laboratory of Infectious Diseases at the RIVM. In 2002 he started his PhD study on the host genetics and genomics of *Bordetella pertussis* in a close collaboration between the Laboratory of Vaccine-preventable diseases (LTR) and the Laboratory of toxicology, pathology and genetics (TOX) at the RIVM under the supervision of Dr. Tjeerd Kimman. After the anthrax-letters in the United States of America in 2001, he volunteered to the national Chemical Biological Radiological and Nuclear (CBRN) response team as gastight suit carrier. In 2007, after completing the study on host genetics and genomics of *Bordetella pertussis* he started working in the CBRN response group as scientific advisor at the Advisory Service for the Inspectorate, Environment and Health (IMG).

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